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Direct analysis in real time mass spectrometry (DART-MS/MS) for rapid urine opioid detection in a clinical setting

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ABSTRACT

Background and aims: Current laboratory methods for opioid detection involve an initial screening with immunoassays which offers efficient but non-specific results and a subsequent liquid chromatography-tandem mass spectrometry (LC-MS/MS) confirmation which offers accurate results but requires extensive sample preparation and turnaround time. Direct Analysis in Real Time (DART) tandem mass spectrometry is evaluated as an alternative approach for accurate opioid detection with efficient sample preparation and turnaround time.

Materials and methods: DART-MS/MS was optimized by testing the method with varying temperatures, operation modes, extraction methods, hydrolysis times, and vortex times. The method was evaluated for 12 opioids by testing the analytical measurement range, percent carryover, precision studies, stability, and method-to-method comparison with LC-MS/MS.

Results: DART-MS/MS shows high sensitivity and specificity for the detection of 6-acetylmorphine, codeine, hydromorphone, oxymorphone, hydrocodone, naloxone, buprenorphine, norfentanyl, and fentanyl in urine samples. However, its performance was suboptimal for norbuprenorphine, morphine and oxycodone.

Conclusion: In this proof-of-concept study, DART-MS/MS is evaluated for its rapid quantitative definitive testing of opioids drugs in urine. Further research is needed to expand its application to other areas of drug testing.

1. Introduction

Globally, it is estimated that 15 million people are addicted to opioids [1]. The surge in opioid prescriptions has led to increased risks of opioid addiction and substance abuse disorders [2]. The abuse of opioids was declared a global health epidemic by the United States Department of Health and Human Services in 2017, leading to an increase in opioid monitoring and vigilance from public health and law enforcement agencies [3]. Between 1999 and 2016, the United States experienced over 630,000 fatal overdoses; many stemming from the misuse of prescription opioids [4]. The rise of synthetic opioids such as fentanyl has fueled what the experts are now calling the "fourth wave" of the opioid epidemic [5]. Consequently, the necessity for closely monitoring patient adherence has become increasingly paramount. The need for efficient and accurate opioid detection is emphasized in pediatric cases where false positive results can lead to false accusations and misguided child protection interventions [6].

Current clinical laboratory methods for opioid detection are not maximally efficient in adapting to the changing landscape of opioid abuse. Traditional approaches to opioid testing involve screening (or presumptive testing) by immunoassays and confirmation (or definitive testing) by liquid chromatography-tandem mass spectrometry (LC-MS/MS). These methods all have their unique set of shortcomings: immunoassays, including immunoassay fentanyl strips, lack specificity and are prone to false-positive and false-negative results while LC-MS/MS methods offer accuracy and sensitivity but are labor intensive and require long analysis time which makes it only appropriate to run in batch mode resulting in a turnaround time of several days [7–10].

Opioid detection and quantification in a time-efficient and accurate manner are critical for opioid addiction interventions as well as patient

Abbreviations: DART-MS/MS, Direct Analysis in Real Time tandem mass spectrometry; %CV, coefficient of variation; % Recovery, percent of recovery; 6MAM, 6-acetylmorphine; MOR, Morphine; COD, Codeine; HC, Hydrocodone; HM, Hydromorphone; OC, Oxycodone; OM, Oxymorphone; NAL, Naloxone; BUP, Buprenorphine; NORBUP, Norbuprenorphine; FENT, Fentanyl; NORFENT, Norfentanyl.

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adherence monitoring. Requiring long turnaround times, current methods for opioid detection have room for improvement to address the growing need for efficient opioid quantification and detection.

Direct analysis in real time (DART) is an ion source that can ionize molecules in a non-contact fashion at ambient pressure. Within the DART, a high voltage needle creates electronically or vibronic excited-state species from inert gases such as helium (Fig. 1). These excited-state species ionize the sample surface molecules. As the inert gas is exiting the DART, a heater coil increases its temperature. The heat helps in the desorption of molecules on the surface of samples placed between the DART and the mass spectrometer inlet [11]. DART enables ionization of samples in native state, bypassing the need for liquid chromatography (LC) that is needed for LC-MS/MS, thus facilitating immediate sample analysis. DART-MS/MS has typically been used in cases of food chemistry applications and it has not been validated for clinical use [12,13]. In this study, a novel approach using Direct Analysis in Real Time (DART) tandem mass spectrometry is validated as a proof-of-concept for the quantitative screening of opioids and opioid-related drugs in urine.

2. Materials and methods

2.1. Reagents and chemicals

LC-MS grade methanol and ethyl acetate were purchased from Thermo Fisher Scientific (Waltham, MA). Water is a clinical laboratory reagent grade water from an in-house deionization system. BG100® β-Glucuronidase (B-one) from Kura Biotec (Los Lagos, Chile). Internal standard solutions including 100 μg/mL 6-acetylmorphine-D6 in acetonitrile and 1.0 mg/mL codeine-D6, norfentanyl-D5, hydrocodone-D6, and morphine-D6 in methanol were purchased from Cerilliant (Round Rock, TX). Standard solutions including 1.0 mg/mL 6-acetylmorphine in acetonitrile and codeine, hydrocodone, hydromorphone, morphine, naloxone, oxycodone, oxymorphone, fentanyl, norfentanyl, buprenorphine and norbuprenorphine in methanol were purchased from Cerilliant (Round Rock, TX). High and low hydro-QCs (morphine-3-β-D-glucuronide and codeine-6-β-glucuronide) were purchased from UTAK (Santa Clarita, CA). Certified drug free urine was purchased from UTAK (Santa Clarita, CA). Liquichek Immunoassay Plus control were purchased from Bio-Rad (Hercules, CA).

2.2. Calibrators, quality controls, IS, and hydrolysis mix preparation

Internal standards stock solution was prepared in methanol with a final concentration of 20,000 ng/mL of (codeine-D6, morphine-D6), and 4000 ng/mL of (buprenorphine-D4, norfentanyl-D5 and 6-acetylmorphine-D6).

Drugs are excreted in the urine as free molecules and/or glucuronide-conjugated compounds. To measure the total concentration of the analyte (by combining certain groups of metabolites into a single species for analysis), it is common practice in clinical toxicology to hydrolyze the glucuronide-conjugated form into the free-form using glucuronidase. The hydrolysis mix was prepared fresh with each use by combining internal standard stock solution, water, and B-one. The stock internal standard solution was first diluted to a final concentration of 2500 ng/mL and 500 ng/mL with water according to the number of samples tested. B-one was then added to the hydrolysis mix in a 38:2 volume-to-volume ratio to the internal standard solution.

Standards were split into three different groups and a working stock solution of 100,000 ng/mL in methanol (50,000 ng/mL for 6-acetylmorphine) was prepared to make calibrators. Group 1 (morphine, codeine, and 6-acetylmorphine), group 2 (oxymorphone, oxycodone, hydromorphone, hydrocodone, and naloxone), and group 3 (norbuprenorphine, buprenorphine, norfentanyl, and fentanyl). A twelve-point calibration curve in drug-free urine was prepared spanning the concentrations 0–1000 ng/mL for group 3 analytes and 6-acetylmorphine and the concentrations 0–2000 ng/mL for the rest of the analytes in groups 1 and 2. Similarly, four levels of quality controls (QCs) were prepared in drug-free urine with the following concentration 10 ng/mL, 25 ng/mL, 250 ng/mL, and 750 ng/mL for 6-acetylmorphine, 25 ng/mL, 50 ng/mL, 500 ng/mL, and 1500 ng/mL for the rest of analytes in groups 1 and 2, and 5 ng/mL, 10 ng/mL, 50 ng/mL, and 500 ng/mL for group 3 analytes. All calibrators and QCs were stored at −20 °C until use.

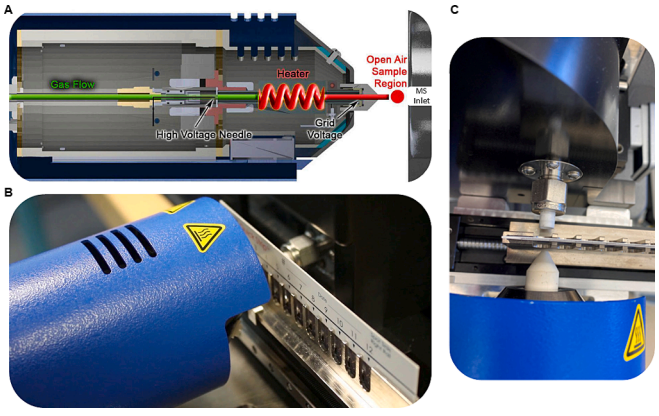

Fig. 1. DART-MS/MS illustration. A. shows how the DART uses a high voltage needle to create metastable ions in the inert gas that can ionize samples placed between the DART and the mass spectrometer. B and C. show the DART installed on the mass spectrometer with the Quickstrip for sample introduction (holds 12 samples) and an automated rail to move between samples.

2.3. Sample preparation

All clinical samples were left over from patient urine samples received for drug confirmation by LC-MS/MS at Yale-New Haven Hospital's Special Chemistry laboratory and were stored at −20 °C until use. Calibrator, QCs and samples were thawed at 4 °C, then an aliquot of 40 μL was mixed with 40 μL of hydrolysis mix and left at room temperature for 1 min before 75 μL of ethyl acetate was added using a multi-dispenser pipette. Samples were vortexed for 10 s and then centrifuged for 5 min at 15,000*g* at room temperature. Finally, 5 μL from the supernatant was spotted on the mesh of the assigned sample number on the Ion Sense DART Quickstrip and left to dry at room temperature before analysis.

2.4. DART-MS/MS conditions

DART-MS/MS was performed using the Ion Sense (*now Bruker*) DART and Waters XEVO TQS MS/MS system operating in positive ion mode (Fig. 1). DART parameters were set to operate at 25 °C using helium gas with a scan mode of 0.8 mm/sec. Analysis was performed using DART ionization in positive ion mode with multiple reaction monitoring with precursor and characteristic product ions specific for each monitored analyte. Two fragment ions per analyte were monitored: one quantifier ion for quantification and one qualifier ion for confirmation using ion ratios. The mass spectrometer collision energies and the cone voltage were set to give optimal signal response from the direct infusion of each analyte in methanol.

For sample introduction, the Ion Sense DART Quickstrip was used which holds 12 samples at a time on fine mesh grids. Samples were spotted using a 3D printed custom-made holder so that the mesh did not come into contact with any surface and was able to evaporate completely before the Quickstrip was analyzed through DART-MS/MS using an automated rail to move between samples and introduce samples to the DART ion source (Fig. 1B, C). Detailed MS/MS conditions for all 12 analytes are listed in Supplemental Table 1.

2.5. Sample preparation and DART optimization

Optimization was performed on all analytes unless mentioned otherwise. DART temperature was optimized using pure standards tested at different DART temperatures from 50 °C to 400 °C at 50-degree increments. Then two different DART operation modes were tested (pulsing and scanning) using morphine and codeine to test the best conditions that will produce the highest signal. In pulsing mode, the DART would align with the center of the Quickstrip mesh between the DART and the mass spectrometer inlet, and the DART would continuously ionize and introduce the sample into the mass spectrometer for 10 s before moving to the next sample. In scanning mode, the DART would scan the Quickstrip mesh from beginning to end at scan rate of 0.8 mm/sec (around 10 s total). Two extraction methods were also tested: "dilute and shoot" using methanol and liquid–liquid extraction using ethyl acetate. The hydrolysis efficiency of the BG100® β-Glucuronidase at room temperature was tested at 1, 5, 10, and 15 min using morphine-3-β-D-glucuronide and codeine-6-β-glucuronide quality controls from UTAK to optimize the time needed to get an optimum level of hydrolysis. Different vortex times for liquid–liquid extraction were tested at 10 s, 1 min, and 3 min using concentrations of 100 ng/mL, 500 ng/mL, and 2000 ng/mL of all analytes.

2.6. DART-MS/MS validation

Analytical measurement range (AMR) was tested using 11 non-zero linearity samples spanning concentrations from 1 ng/mL to 2000 ng/mL. These were extracted, run in triplicates, and quantified on the DART-MS/MS using the procedures mentioned above. The acceptability criteria used was a <20 % coefficient of variation (CV) and recovery within 80–120 %.

Carryover was tested by running the highest calibration curve point and the second lowest QC in the following order low QC, high calibration, low QC, low QC, low QC, repeated three times. The acceptability criteria used was % deviation of the QC after high calibration from QC before the high calibration within 20 %. Percent carryover was measured using the following formula: % Carryover = [(QC after- QC before)/Cal high] × 100. Carryover results were validated by checking the second sample of every analyte with a concentration above the AMR.

Three types of precision assays were completed. Intraday precision was measured across all four quality control samples spanning the AMR that were extracted and run six times on the same day. Inter-day precision was measured across the four levels that were extracted and run 14 times over a period of five days. Hydrolysis precision was measured using the high and low hydro-QCs with morphine-3-β-D-glucuronide and codeine-6-β-glucuronide that were run for six times over six days.

Analytical specificity was measured by running patient urine samples free of drugs being tested through the Liquichek Immunoassay Plus control that contains 88 common endogenous and exogenous analytes (Supplemental Table 3) and by running analytes that are known to have the same molecular weight as the ones in the panel to see if they cause ion ratios to flag.

Stability was tested over five hours either in the extraction vial or spotted on the Quickstrip. For the extraction vial stability, a 5 μL sample was taken from the supernatant, spotted on the Quickstrip, dried and run every hour for five hours. For the Quickstrip stability, five samples were spotted on the quick strip, left on the bench at room temperature and one sample was measured each hour for five hours. All analytes were prepared in 100 ng/mL samples before extraction.

Method-to-method comparison was performed using an 11-point standard, excluding the blank sample, to generate calibration curves for 6-acetylmorphine (6MAM), codeine, hydrocodone, hydromorphone, morphine, naloxone, oxycodone, and oxymorphone. A total of 179 samples were analyzed on DART-MS/MS using the calibration curve and compared to data from an in-house LC-MS/MS method for drug quantification. Averages of internal standards of the calibrators for each analyte were calculated and samples with an internal standard area count below 20 % of the analyte's calibrator average were excluded due to severe ion suppression (affecting DART-MS/MS). The correlation between the two methods was calculated with the R-value and slope. Clinical sensitivity and specificity of the DART-MS/MS were calculated by using the LC-MS/MS values as true positives and negatives.

2.7. Statistical analysis

All statistics were performed using GraphPad Prism Version 9 and Excel.

3. Results

3.1. Method development

First, we tested different DART parameters using pure standards. Across 50 °C intervals between temperatures from 50-400 °C, the maximum area of the DART chromatogram was observed for all analytes were between 250 and 300 °C, and we chose 250 °C (Fig. 2B). Testing the DART sample introduction mode, either via pulsing or scanning, was found to favor scanning to optimize the area of the DART chromatogram for both codeine and morphine (Fig. 2C). Next, we looked at different extraction and hydrolysis conditions. We tested different extraction methods at two concentrations (200 ng/mL and 400 ng/mL) for all analytes and we found that liquid–liquid extraction using ethyl acetate produced higher area counts with lower noise (Fig. 2D). Testing the hydrolysis efficiency of BG100® β-Glucuronidase at room temperature on morphine and codeine at 200 ng/mL and 1000 ng/mL showed no significant difference between samples left for 1 min up to 15 min at room temperature (Fig. 2E). Similarly, we didn't see a significant difference between samples vortexed for 10 s, 1 min, and 3 min in all analytes at concentrations of 100 ng/mL, 500 ng/mL, and 2000 ng/mL (Fig. 2F).

3.2. Validation

The analytical measurement range (AMR) evaluation determined clinically acceptable lower and upper limits of quantification based on CV<20 % and recovery within 80–120 %. Fentanyl AMR spanned concentrations from 3 to 1000 ng/mL, while 6MAM, buprenorphine, norbuprenorphine and norfentanyl AMR spanned concentrations from 5 to 1000 ng/mL. Naloxone AMR spanned concentrations from 10 to 1000 ng/mL. The rest of the opioids lower limit of quantification ranged between 5 and 10 ng/mL but were quantified with high precision and accuracy up to 2000 ng/mL. Table 1 and Supplemental Table 2 shows AMR for all analytes along with the percent recovery and CV for each opioid. Next, we tested carryover using highest and lowest calibrators and all 12 analytes showed a carryover of less than 0.5 % with up to 2000 ng/mL and a less than 20 % deviation measured as %CV (Table 4). Moreover, none of the samples that had a concentration above AMR showed any carryover in the subsequent sample.

Intraday and interday precision conducted across four concentrations of quality controls of all 12 analytes show a CV less than 20 %, except for norbuprenorphine where interday precision at the lowest quality control, 5 ng/mL showing a %CV of 33.3. Interday precision conducted on the low and high hydrolysis quality controls also showed %CVs of less than 20 % for both morphine and codeine. We also calculated accuracy using the low and high hydrolysis quality controls using values from LC-MS/MS for expected concentrations and % accuracy ranged between 94 and 114 %. Table 2 shows the mean, standard deviation (SD) and coeffect of variation (CV) for all 12 analytes at the 4 quality control levels in addition to the % accuracy for the hydrolysis low- and high-quality controls.

Stability of samples in the extraction vial showed low sample degradation over five hours at room temperature with the percent

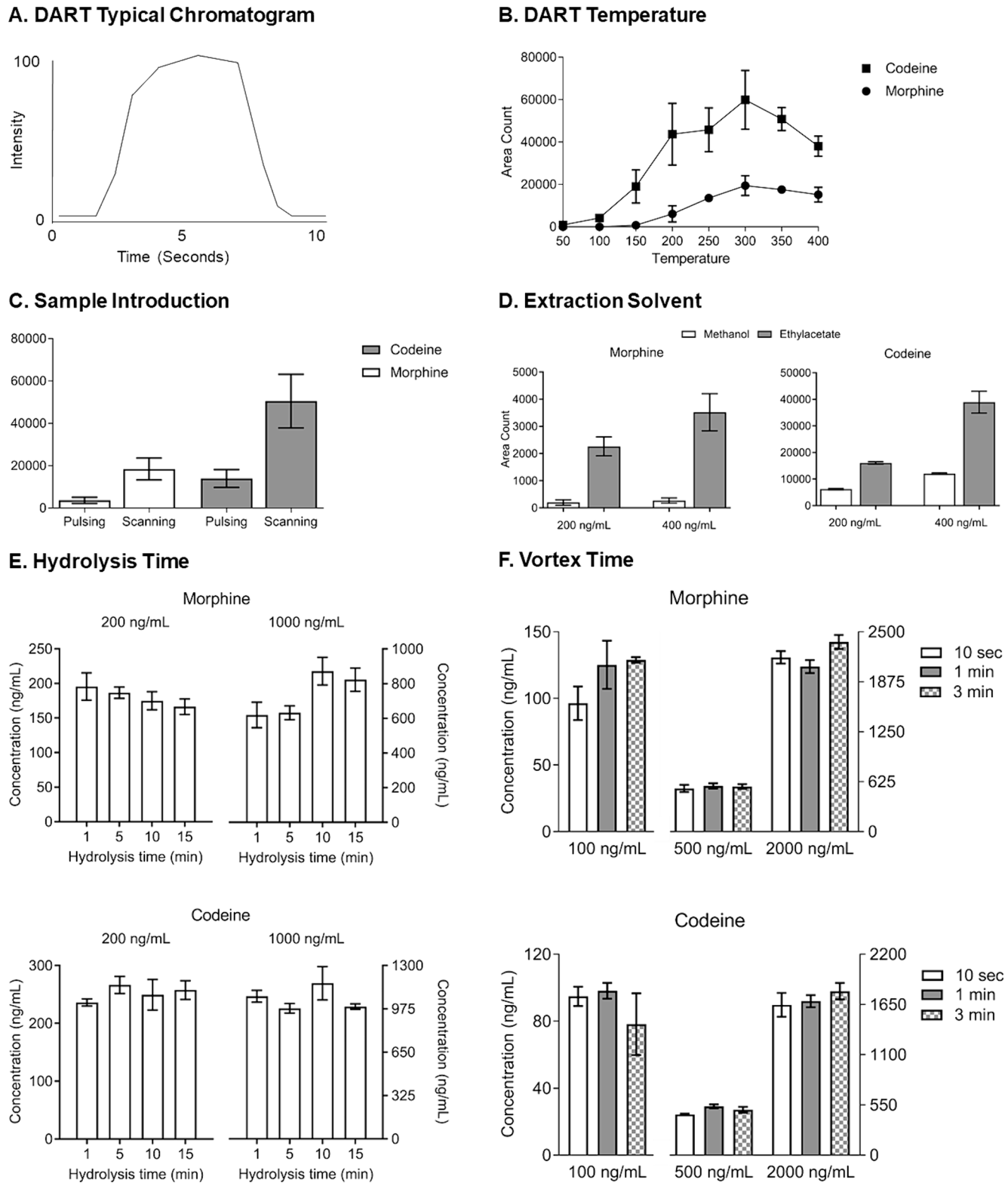

Fig. 2. DART-MS/MS method development. Showing A. typical DART-MS/MS chromatogram, B. effect of DART temperature on area count, C. testing the two different DART modes of sample introduction (pulse and scan), D. testing two different extraction methods (liquid–liquid extraction with ethyl acetate and dilute and shoot with methanol), E. testing the room temperature fast acting hydrolysis enzyme, and F. testing the vortex time effect on recovery.

recoveries for all 12 analytes ranging between 82 and 114 %. Stability of samples on the Quickstrip similarly showed low sample degradation over five hours at room temperature with the percent recoveries of all 12 analytes ranging between 83 and 113 % (Table 3).

Analytical specificity was measured by running patient urine samples free of analytes being tested through the Liquichek Immunoassay Plus control that contains 88 common endogenous and exogenous analytes (Supplemental Table 3) and all 24 MRM channels monitored for the 12 analytes showed minimal signal below the limit of quantification. Next, we tested pain drugs that are known to have the same molecular

Table 1
DART-MS/MS Analytical Measurement Range for Analytes with Lower and Upper Limits of Quantification, Coefficient of Variation, and Percent Recovery.

	Lower Limit of Quantification			Upper Limit of Quantification		
Analyte	LLOQ ng/mL	% CV	% Recovery	ULOQ ng/mL	% CV	% Recovery
6MAM	5	11.0	116.7	1000	5.7	102.2
Morphine	10	16.6	100.7	2000	8.0	93.9
Codeine	10	14.3	81.3	2000	9.9	96.4
Hydrocodone	5	8.1	103.3	2000	5.6	100.1
Hydromorphone	5	4.0	103.3	2000	1.8	108.4
Oxycodone	5	6.5	107.3	2000	7.9	109.4
Oxymorphone	5	9.0	105.3	2000	12.8	101.5
Naloxone	10	6.0	110.3	1000	8.4	93.8
Buprenorphine	5	11.2	82.0	1000	8.7	103.8
Norbuprenorphine	5	6.6	92.7	1000	13.4	95.7
Fentanyl	3	10.6	94.7	1000	3.3	95.8
Norfentanyl	5	11.8	90.0	1000	3.6	99.2

Abbreviations: LLOQ – Lower Limit of Quantification, ULOQ – Upper Limit of Quantification, %CV – Coefficient of Variation, % Recovery – Percent of Recovery.

weight as the ones in the panel. Testing pure hydromorphone, hydrocodone and naloxone flagged the ion ratios for morphine, codeine, and 6MAM respectively and vice versa. A more comprehensive specificity testing was done within the method-to-method comparison where we tested many urine samples that are negative for each analyte.

Method-to-method comparison was performed between the DART-MS/MS and the LC-MS/MS with 179 clinical samples. Among the 179 samples, hydrocodone had the least number of positive samples reported by LC-MS/MS with 15, while morphine had the most reported positive samples reported by LC-MS/MS with 63. Table 5 characterizes the number of samples and the number of positive samples for each analyte as well as the R value, slope, clinical sensitivity, and specificity for all 12 analytes. Buprenorphine, norfentanyl, and hydrocodone all had R-values of 0.99 while morphine, codeine, hydromorphone, naloxone, and fentanyl all had R-values above 0.9. 6MAM, oxymorphone, oxycodone, and norbuprenorphine had the lowest R-values ranging from 0.74 to 0.89. Fentanyl, norbuprenorphine, and hydrocodone had slopes ≤ 1.1 and 6MAM, codeine, hydromorphone, oxycodone, naloxone, buprenorphine, and norfentanyl all had slopes ≤ 1.25. Morphine had the highest slope at 1.33. We observed that for some of the analytes such as oxycodone, oxymorphone, morphine, and 6MAM, one or two outlying points may have skewed the R value and slope.

Fentanyl, norfentanyl, buprenorphine, and hydromorphone had high sensitivity and specificity while naloxone, codeine, oxymorphone, 6MAM, hydrocodone, and norbuprenorphine had excellent sensitivity and specificity. One false positive for 6MAM and both false negatives for naloxone had either LC-MS/MS concentration or DART-MS/MS concentration within 10 % of the positive cut-off for the analyte. One false negative for 6MAM had a DART-MS/MS value within 30 % of the positive cut-off for the analyte which was 10 ng/mL. Morphine and oxycodone had lower sensitivity and specificity, the poorest of which was the specificity of morphine at 0.75.

Table 2
Intraday and Interday Precision for DART-MS/MS Across Four Levels of Quality Control.

	Quality Control 1			Quality Control 2			Quality Control 3			Quality Control 4		
	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
Analyte	Intraday Precision											
6MAM	11.0	1.2	10.7	25.1	2.8	11.3	240.4	12.2	5.1	812.0	78.3	9.6
MOR	23.8	4.3	18.3	55.3	8.2	14.8	512.7	43.5	8.5	1565.3	171.3	10.9
COD	22.7	3.2	14.3	46.8	5.5	11.7	472.6	29.0	6.1	1600.7	200.1	12.5
HC	23.3	1.8	7.7	54.2	2.5	4.6	522.1	22.3	4.3	1542.4	80.7	5.2
HM	22.6	2.6	11.6	49.7	4.0	8.0	513.8	47.9	9.3	1610.1	153.4	9.5
OC	24.0	3.0	12.4	54.0	2.5	4.6	508.8	12.9	2.5	1580.2	123.0	7.8
OM	22.2	4.0	18.3	51.4	1.6	3.1	524.1	66.6	12.7	1617.7	181.4	11.2
NAL	20.4	3.3	16.3	51.0	4.0	7.9	453.3	31.2	6.9	1789.7	329.7	18.4
BUP	5.8	0.6	9.8	11.1	0.9	7.9	111.9	2.6	2.4	507.4	45.3	8.9
NORBUP	4.6	0.8	16.5	10.5	1.2	11.3	89.9	14.6	16.2	466.3	44.4	9.5
FENT	5.2	0.7	14.0	10.0	1.2	12.2	100.9	12.6	12.5	465.4	46.5	10.0
NORFENT	5.1	0.7	13.3	10.2	0.8	8.0	99.8	11.1	11.1	458.3	47.1	10.3
	Interday Precision											
6MAM	11.0	1.1	10.0	23.7	1.2	5.2	241.1	17.4	7.2	779.1	62.2	8.0
MOR	19.2	2.4	12.5	47.6	5.4	11.4	475.1	55.2	11.6	1486.8	139.9	9.4
COD	21.2	3.0	14.2	52.4	5.2	9.9	498.6	23.5	4.7	1566.5	40.2	2.6
HC	21.1	2.4	11.5	47.2	3.9	8.4	481.8	43.6	9.0	1605.1	82.9	5.2
HM	20.9	2.8	13.2	48.4	4.3	8.9	465.1	68.2	14.7	1510.3	212.6	14.1
OC	20.2	2.6	12.8	50.3	5.8	11.5	466.3	63.8	13.7	1484.6	189.9	12.8
OM	21.7	1.7	7.7	45.4	2.9	6.4	459.3	41.1	8.9	1571.5	157.7	10.0
NAL	23.1	0.5	2.3	54.2	5.8	10.6	527.1	50.8	9.6	1571.4	168.2	10.7
BUP	5.4	0.8	14.9	10.4	1.2	11.2	106.7	7.9	7.4	484.9	44.2	9.1
NORBUP	6.5	2.2	33.3	10.5	1.2	11.2	88.3	10.3	11.6	475.2	62.1	13.1
FENT	5.0	0.7	14.0	10.4	1.4	13.0	97.9	12.3	12.6	448.8	38.9	8.7
NORFENT	5.2	0.6	12.0	10.2	0.8	8.1	102.7	8.7	8.4	477.0	59.3	12.4
	Hydrolysis Quality Control Accuracy and Precision											
	Low Quality Control				Low Quality Control							
	Mean	SD	%CV	% Accuracy	Mean	SD	%CV	% Accuracy				
MOR	187.6	31.8	16.9	113.4	936.2	180.7	19.3	94.1				
COD	201.0	22.0	10.9	98.1	795.5	108.2	13.6	95.7				

Abbreviations: 6MAM – 6-acetylmorphine, MOR – Morphine, COD – Codeine, HC – Hydrocodone, HM – Hydromorphone, OC – Oxycodone, OM – Oxymorphone, NAL – Naloxone, BUP – Buprenorphine, NORBUP – Norbuprenorphine, FENT – Fentanyl, NORFENT – Norfentanyl. The mean concentration, standard deviation (SD) and coefficient of variation (%CV) for four levels of quality controls are described for intraday and interday precision analysis. Hydrolysis data for low quality control accuracy and precision is shown.

Table 3
Stability of Analytes in Vial vs. On Strip: Percent Recovery at 1 to 5 Hours Post-Preparation.

Analyte		% Recovery 1 h	2 h	3 h	4 h	5 h
6MAM	In Vial	92.4	115.4	88.4	92.6	88.4
	On Strip	100.7	93.9	93.4	97.6	92.5
Morphine	In Vial	107.6	83.2	93.5	93.5	81.9
	On Strip	85.4	85.2	99.1	89.6	89.9
Codeine	In Vial	97.6	96.9	101.2	101.2	101.4
	On Strip	105.3	97.9	103.4	97.5	89.8
Hydromorphone	In Vial	117.7	86.8	103.8	103.8	113.7
	On Strip	81.1	99.3	82.2	84.0	82.7
Oxymorphone	In Vial	115.6	81.6	101.8	101.8	103.4
	On Strip	84.2	116.4	83.8	86.1	90.9
Hydrocodone	In Vial	87.1	90.6	103.2	106.6	101.0
	On Strip	87.9	83.2	82.0	83.7	84.8
Oxycodone	In Vial	91.1	87.8	106.3	100.1	101.4
	On Strip	83.1	90.1	85.5	86.9	96.3
Naloxone	In Vial	94.0	89.5	116.9	105.6	94.9
	On Strip	102.3	110.6	111.8	110.5	113.1
Norbuprenorphine	In Vial	80.9	90.6	88.5	96.3	85.5
	On Strip	93.8	114.3	90.5	85.5	86.7
Buprenorphine	In Vial	114.1	103.2	95.3	110.7	97.5
	On Strip	87.8	91.4	91.8	103.3	98.6
Norfentanyl	In Vial	112.4	103.5	101.8	96.4	109.7
	On Strip	82.4	102.1	109.5	88.2	105.8
Fentanyl	In Vial	83.1	115.1	105.6	100.5	82.4
	On Strip	98.0	85.9	86.3	88.7	83.1

Abbreviations: % Recovery – percent recovery.

Table 4
Analyte Carryover and Deviation in DART-MS/MS.

Analyte	% carryover	% deviation of QC_{after} from QC_{before}
6MAM	−0.3	10.5
Morphine	0.2	8.7
Codeine	−0.4	11.5
Hydrocodone	0.1	2.7
Hydromorphone	0.1	3.9
Oxycodone	−0.1	5.4
Oxymorphone	0.2	8.2
Naloxone	0.1	4.2
Buprenorphine	−0.2	9.2
Norbuprenorphine	0.03	1.6
Norfentanyl	−0.3	11.9
Fentanyl	−0.3	11.2

Abbreviations: % carryover – percent carryover, % deviation – percent deviation

The ion ratios data collected during the method-to-method validation was used to gain a deeper understanding at how ion ratios can help add a layer of specificity in the case of DART-MS/MS which will require certain samples to be confirmed by LC-MS/MS. Data of ion ratios from three sets of isobaric analytes that can cause interference between each other (morphine and hydromorphone, codeine and hydrocodone, 6MAM and naloxone) are illustrated in Fig. 3.

By comparing samples that were hydromorphone and morphine positive, all samples positive for hydromorphone and negative for morphine that had a detectable morphine peak had morphine ion ratios outside the limits. And similarly, all except three of the samples with hydromorphone peaks detected that were morphine positive and hydromorphone negative had hydromorphone ion ratios outside the limits. Moreover, all except for one of the samples positive for both morphine and hydromorphone had either morphine, hydromorphone or both ion ratios out of the acceptable limits. Out of the 76 samples positive for morphine, hydromorphone, or both, 47 samples had a presumptive positive DART-MS/MS concentration as one or both ion ratios outside the limits and would be repeated by LC-MS/MS. Of the samples which were presumptive positive and repeated by LC-MS/MS, 28 were confirmed negative.

By comparing samples that were hydrocodone positive and codeine negative, all samples yielded codeine ion ratios outside of acceptable limits. Samples that were hydrocodone negative and codeine positive yielded all hydrocodone ion ratios outside the acceptable limits and five samples with codeine ion ratios outside of acceptable limits. Out of the 39 samples positive for codeine, hydrocodone, or both, 15 had a presumptive positive DART-MS/MS concentration as ion ratios outside the limits and must be repeated by LC-MS/MS of which 13 were confirmed negative.

By comparing samples positive for both 6MAM and naloxone, none had naloxone ion ratios outside of acceptable limits and five samples had 6MAM ion ratios outside of acceptable limits. For samples negative for 6MAM and positive for naloxone, all samples yielded 6MAM ion ratios outside the acceptable limits, and none had naloxone ion ratios outside of acceptable limits. For samples positive for 6MAM and negative for naloxone, all samples yielded naloxone ion ratios outside the acceptable limits, and none had 6MAM ion ratios outside of acceptable limits. Out of the 40 samples positive for 6MAM, naloxone, or both, 13 had a presumptive positive DART-MS/MS concentration as ion ratios outside the limits and must be repeated by LC-MS/MS of which 8 were confirmed negative.

Table 5
Method to Method Comparison of Analytes on DART-MS/MS: R-value, Slope, Sensitivity, and Specificity.

Analyte	Total # of Samples	# of positive samples (LC-MS-MS)	% Positive	# of samples removed due to low internal standard by DART	# of samples removed due to ion ratios outside of cut-off	R value	Slope	Positive Cut Off	Sensitivity	Specificity
6MAM	129	20	15.5	6	27	0.74	1.16	≥10	0.89	0.99
MOR	129	63	48.8	11	22	0.90	1.33	≥50	0.98	0.75
COD	129	33	25.6	18	17	0.95	1.24	≥50	0.96	0.98
HM	146	26	17.8	20	37	0.91	1.16	≥50	1.00	1.00
OM	146	52	35.6	20	8	0.82	0.68	≥50	0.96	0.97
HC	146	15	10.3	23	18	0.99	1.06	≥50	1.00	0.99
OC	146	52	35.6	23	1	0.84	1.16	≥50	0.89	0.91
NAL	146	32	21.9	23	3	0.96	1.14	≥50	0.93	1.00
NORBUP	47	30	63.8	2	4	0.89	1.07	>5	1.00	0.83
BUP	50	33	66.0	2	2	0.99	1.23	>5	1.00	1.00
NORFENT	47	29	61.7	0	0	0.99	1.17	>5	1.00	1.00
FENT	50	30	60.0	0	0	0.90	1.07	>3	1.00	1.00

Abbreviations: Total # of Samples – Number of samples tested for analyte, # of positive samples (LC-MS/MS) – Number of samples with a positive LC-MS/MS result, % Positive – percent of samples positive by LC-MS/MS, # of samples removed due to low internal standard – Number of samples removed due to low internal standard of DART-MS/MS result, # of samples removed due to ion ratios outside of cut-off – Number of samples removed due to DART-MS/MS ion ratios being out of acceptable limits.

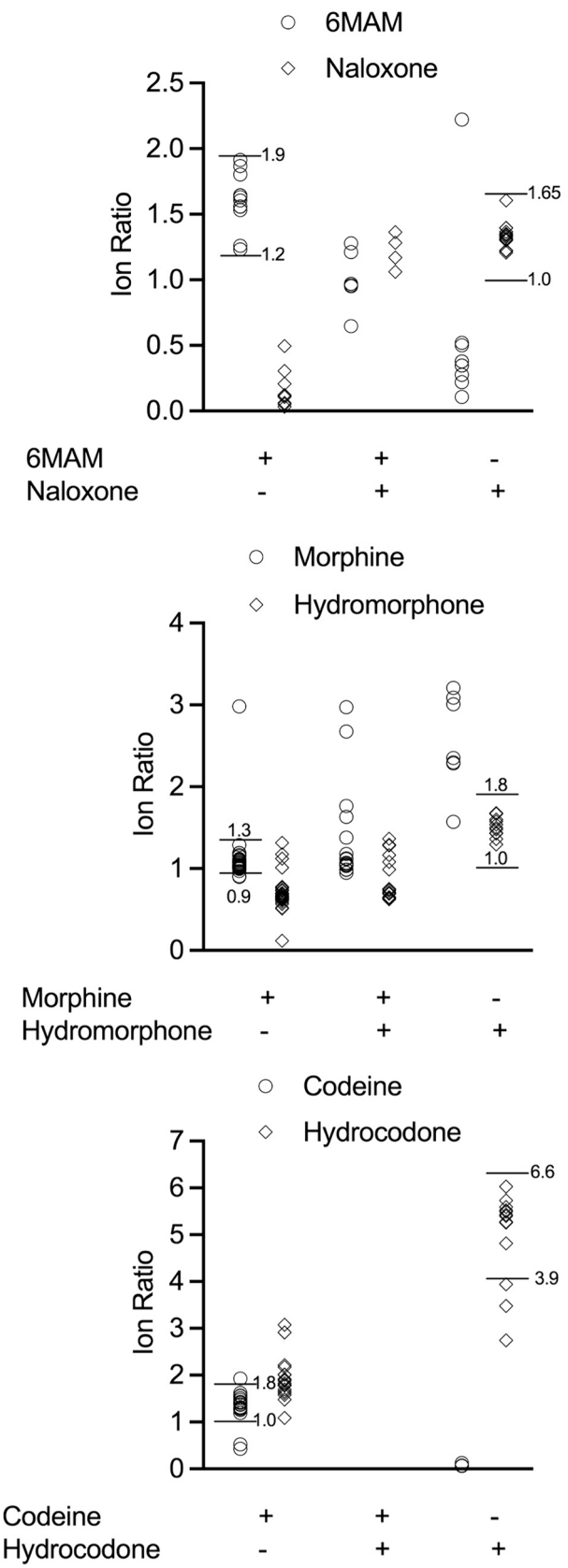

Fig. 3. DART-MS/MS ion ratios for isobaric compounds. Illustrates data of ion ratios from three sets of drugs that can cause interference between each other (morphine and hydromorphone, codeine and hydrocodone, 6MAM and naloxone). Cutoff values represented by the black line are calculated using ion ratios from calibration curve.

4. Discussion

In this study we have developed and evaluated a Direct Analysis in Real Time tandem mass spectrometry (DART-MS/MS) method for the quantification of opioids and opioid-related drugs in urine for clinical use. We have reported detailed DART specific parameters like DART temperature, sample introduction (scanning vs pulsing modes) that will help guide future scientists who aim to develop more assays using DART-MS/MS.

While DART-MS/MS bypasses the time-consuming chromatography step, urine drug testing still requires tedious time-consuming extractions. To overcome this hurdle, we developed a fast extraction method that utilizes a genetically modified room temperature fast acting β-Glucuronidase enzyme coupled with liquid–liquid extraction with ethyl acetate. The extraction time was cut down from more than one hour for a typical LC-MS/MS method to less than ~ 7 mins. Moreover, the DART-MS/MS run time requires around 10 s per sample that is much faster than the a typical gas or liquid chromatography mass spectrometry methods that averages around 10–20 mins run per sample [14–21].

In the DART-MS/MS method validation we showed that the method can reach clinically required low and high concentrations for all 12 drugs and metabolites tested. Moreover, precision studies showed good inter and intra-day precision with % CV ranging between 2 and 18 %. The validation data also recognized two DART-MS/MS unique criteria in terms of carryover and sample stability. For carryover, the DART-MS/MS by design does not have parts that comes in contact with the actual sample that might contaminate the following sample, and this was clearly shown in the very low % carryover results reported in all tested compounds ranging between −0.4 to 0.1 %. For sample stability, in a typical LC-MS/MS we transfer extracts into a mass spectrometer vial where we measure post-extraction stability. The unique thing about the DART-MS/MS is that there is no vial, instead there is a QuickStrip that holds up to 12 samples. Post-extraction stability experiments for LC-MS/MS are tested over the course of multiple days which is necessary for these methods since it might require a few days for a run to finish, data analyzed and ultimately to notice that a sample rerun is needed. Since DART-MS/MS analysis can be completed within a few minutes, with the results returned within the hour, we designed a stability experiment in both the extraction vial and on the Quickstrip over the course of 5 h. Samples that remained in extraction vials and on the Quickstrip yielded a high percent of recovery across all compounds tested, indicating high sample stability in both the vial and the Quickstrip that could produce accurate results even when not tested immediately.

The method-to-method comparison against LC-MS/MS and performance analysis for DART–MS/MS on opioid detection and quantification is presented. In total, 179 samples were tested and analyzed across 12 analytes: 6MAM, morphine, codeine, hydromorphone, oxymorphone, hydrocodone, oxycodone, naloxone, norbuprenorphine, buprenorphine, norfentanyl, and fentanyl with a total of 1311 unique test results. A total of 148 test results (out of 1311) were excluded due to a low internal standard below 20 % of the average internal standard of the calibrators when measured by DART-MS/MS. Another 140 test results were also excluded since they were positive but had an ion ratio outside of acceptable limits (would have been directed to re-run on LC-MS/MS). 1023 of the test results were considered for method-to-method comparison and DART-MS/MS correctly detected the presence or absence of opioids based on each compound's respective positive cut-offs in 987 runs (96.5 %).

The R value and the slope for the correlation between the LC-MS/MS opioid concentration value and the DART-MS/MS opioid concentration value were calculated. We observed that for some of the analytes such as oxycodone, oxymorphone, morphine, and 6MAM, one or two outlying points may have skewed the R value and slope. The sensitivity and specificity values for immunoassays testing various analytes differ but are generally comparable or worse as compared to DART-MS/MS values [22,23,24]. Immunoassays have especially been found to be unreliable

in detecting semisynthetic opioids such as buprenorphine, oxycodone, hydrocodone, oxymorphone, and hydromorphone, and thus tend to lack specificity and sensitivity [25].

Analysis of our test results, especially the ones where DART-MS/MS failed to characterize accurately, can yield important insights regarding its strengths and shortcomings. Fentanyl, norfentanyl, buprenorphine, and hydromorphone all showed 100 % sensitivity and specificity in this study. Of the sample runs that failed, two were false positives for norbuprenorphine, but both samples also were true positive for buprenorphine. On further examination, the norbuprenorphine values were close to the cut-off by LC-MS/MS, so these differences are expected around cutoff values and, in this case, DART-MS/MS was more clinically concordant. Similarly, two false negative samples for naloxone were both detected with concentrations close but lower than the used cut-off value of 50 ng/mL. There were one false negative and one false positive for 6MAM, both samples were detected in both methods around the cut-off value (10 ng/mL). One false positive for hydrocodone with a concentration just above the cut-off value of 50 ng/mL. One false negative and one false positive for codeine, the false negative has a detectable codeine with result lower than the cut-off value of 50 ng/mL and the false positive was a sample that also flagged a false positive for morphine which could indicate a sample-related random error or interference. Two false negatives and two false positives for oxymorphone, one of the false negative samples was detectable with concentration just below the cut-off value and the other was below LOQ with oxycodone result also below LOQ as well. For the two false positives, one of them was also positive for oxycodone which would not have affected clinical interpretation, while the other was negative for oxycodone, but the sample was detectable by LC-MS/MS lower than the cut-off value. Oxycodone and morphine were the only two analytes that had high number of samples that the DART-MS/MS failed to characterize accurately, with seven false positives and five false negatives for oxycodone and ten false positives and one false negative for morphine.

We also evaluated DART-MS/MS's ability to distinguish between isobaric compounds, which have the same mass-to-charge ratio of parent compounds, via ion ratios, as is the case for isobaric pairs 6MAM and naloxone, morphine and hydromorphone, and codeine and hydrocodone (Fig. 3). To facilitate the separation and differentiation between positive and negative values on these isobaric pairs, the acceptability of ion ratios was analyzed. DART-MS/MS ion ratio analysis shows that it can handle this differentiation as the ion ratios of samples positive for both analytes, and samples positive for one of two analytes show distinct ion ratio ranges that can be used for specific drug detection. Separation of these isobaric compounds have previously been attempted by ion mobility in tandem with mass spectrometry with urine samples in proof-of-concept studies, but a recent study found that morphine / hydromorphone and codeine / hydrocodone were pairs of isobaric compounds that were classified as inseparable while moderate separation was achieved with 6MAM and naloxone [26,27]. So, implementing ion ratios like we did is key for reporting confident results, and reflexing results to LC-MS/MS when the ratios flag.

Overall, DART-MS/MS shows high sensitivity and specificity and a strong capability for opioid detection for most analytes tested including 6MAM, codeine, hydromorphone, oxymorphone, hydrocodone, naloxone, buprenorphine, norfentanyl, and fentanyl. Analysis of inaccurate results shows that DART-MS/MS is susceptible to ion interference and the detection for a given analyte may be vulnerable to endogenous metabolites from the presence of high concentrations of other analytes. This effect seems to be problematic for some of the analytes tested but not all of them. Among those that DART-MS/MS is a good candidate for detection, fentanyl and norfentanyl are among the most pertinent as ED cases of norfentanyl and fentanyl overdose require speedy and accurate detection that neither current immunoassays nor LC-MS-MS methods can satisfy.

This study shows that DART-MS/MS cannot fully replace an LC-MS/MS system since 22 % of the results would still require confirmation by LC-MS/MS. DART-MS/MS is proposed as a first-line definitive method that would require only 22 % of the results to be reflexed to full LC-MS/MS rather than all presumptive results, which would be the case with immunoassays. Though the DART-MS/MS method tested in this study has limitations for compounds such as morphine, norbuprenorphine, and oxycodone, its excellent performance with norfentanyl and fentanyl show that it can fill this need where immunoassays fall short. In addition, DART-MS/MS may be an effective tool to screen for compounds that do not have immunoassays available such as xylazine or fentanyl analogues. Rapid development and deployment of this tool for new drugs and emerging threats may help provide another testing option for laboratories to deal with a new overdose crisis. It could also help provide more rapid results in the setting of presumptive positive pediatric cases where child protective services may be involved, instead of waiting for days for definitive testing by LC-MS/MS.

Theoretically, DART-MS/MS could be run in real-time for the purpose of drugs of abuse detection to support emergency medicine applications. Lab technicians could run these tests individually and it would take around 10 min from the start of a sample's extraction to the release of the results. While this process can be automated using software like "Ascent" (Indigo Bioautomation, Carmel, IN), the proposed method evaluated here requires manual review. We propose a method for running single samples: a single strip can accommodate two QCs, two calibration points, one blank, and the sample. This fills up half of the strip and would take around 1 min to run. Additionally, a full calibration curve will be built every day and for each sample two points of calibration can be run for verification.

In this proof-of-concept study, we demonstrated that DART-MS/MS has the potential to be used as an efficient tool for rapid definitive testing for some opiates, while flagging results it could not resolve to be tested by LC-MS/MS. This has the potential for saving hours of unnecessary testing by LC-MS/MS, when a definitive test can be obtained in seconds on DART-MS/MS. Further research and development are needed to overcome the DART limitations discussed in our paper and to expand its applications to other areas of drug testing.

Author contributions

The corresponding author takes full responsibility that all authors on this publication have met the following required criteria of eligibility for authorship: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list.

CRediT authorship contribution statement

Ibrahim Choucair: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Emily Shang:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Minh Nguyet Tran:** Writing – review & editing, Validation, Methodology, Formal analysis. **Gina Cassella-McLane:** Writing – review & editing, Supervision, Resources, Project administration, Formal analysis. **Joe M. El-Khoury:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

For the duration of the research study, we received the Direct Analysis in Real Time (DART) technology from IonSense, Inc. (now owned by Burker) free of charge. Despite this support, the authors maintained full independence in the design, execution, analysis, and interpretation of the research. No financial support was provided, and IonSense, Inc. (now Bruker) had no role in the decision to publish or preparation of the manuscript. The authors have no other competing interests to declare that are relevant to the content of this article.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.119939.

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