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Validated method for the analysis of 22 illicit drugs and their metabolites via liquid chromatography tandem mass spectrometry (LC-MS/MS) in illicit drug samples collected in Chicago, IL

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ABSTRACT

Drug checking services are being utilized worldwide to provide people who use drugs information on the composition and contents of their drugs as a tool for harm reduction and accidental overdose prevention. Existing drug checking services use a variety of techniques including immunoassay strips and spectroscopic techniques like FTIR and Raman. Few services utilize LC-MS based methods for primary or secondary analysis and few methods exist for direct analysis of illicit drugs. To address this, an LC-MS/MS method was developed for 22 illicit drugs and cutting agents using LC-MS/MS with application to 124 illicit drug samples that were collected from Chicago, IL. Samples were also analyzed using fentanyl and benzodiazepine immunoassay test strips. Fentanyl test strips gave a positive result for 86% of samples with only one sample showing a positive result on a benzodiazepine test strip. LC-MS/MS analysis of samples show that opioids were the most commonly quantified in 96% of samples, followed by stimulants at 12% and benzodiazepines at 1%. Fentanyl was measured in 91% of samples, co-occurring with heroin in 58% of opioid-containing samples. A comparison of the gold-standard LC-MS/MS results to fentanyl test strips shows a high level of accuracy for the fentanyl test strips, with just 5% of samples being classified as false negatives and no false positives. These results demonstrate the strengths and benefits of LC-MS/MS when incorporated as a secondary analysis tool for drug checking.

Introduction

Deaths from accidental overdoses have been on the rise in the United States for more than 20 years. In 2021, the annual number of drug overdoses topped 100,000 for the first time with a significant portion of deaths associated with synthetic opioids, namely fentanyl [1]. Between 2013 and 2019 overdose deaths involving synthetic opioids increased over 1,000 % and accounted for 60 % of all overdoses in 2021 [2,3]. Reducing the risk and harm associated with drug use, including prevention of fatal overdoses, is dependent on evidence-based approaches implemented at the clinical, regulatory, carceral, and community levels [4]. At the community level, emerging tools for people who use drugs are supervised injection sites and drug checking services. Drug checking services utilize a variety of tools and techniques to provide information on drug composition along with additional resources to enable informed decision-making in people who use drugs, with an overall goal to reduce the harm associated with drug use [5]. Data generated through drug checking also serve as important information for monitoring regional and local drug trends to inform drug users and institutional health services of both emerging and continuing drugs present in street samples. Information on drug composition is typically measured using a variety of techniques, including immunoassay strips, reagent testing, spectroscopy techniques such as FTIR and Raman, and more recently mass spectrometry techniques like GC- or LC-MS [6,7].

The techniques utilized vary in their cost, ease of use, portability, and the type of results generated. Colorimetric reagent kits are inexpensive and easy to use but fail to provide detailed information on drug composition or concentration and often measure only bulk content, or those compounds present at percent-level concentrations [8,9]. FTIR and Raman also only measure bulk content of a drug with limits of detection in the low percent weight range but utilize spectral libraries to provide relatively accurate identifications of the drug(s) present [7,10,11]. Versions of these instruments are available in field-friendly portable formats that can be employed by mobile drug checking services. Immunoassay strips, especially those designed for fentanyl screening, have emerged as both a sensitive and selective tool for rapidly testing drugs. These strips, while initially designed for analysis of drugs in urine, can be used by dissolving a small quantity of drug into

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water [12,13]. Immunoassay fentanyl test strips are now part of routine drug checking services across the country and worldwide and have been found to be a useful tool for changing drug use behavior and to prevent accidental overdoses [14–17].

Less commonly used in drug checking services as part of a harm reduction approach are GC- and LC-MS based techniques. These techniques are routinely used in clinical and regulatory laboratories for screening biological samples and seized drugs due to the high level of specificity and sensitivity of generated results [18–23]. A vital benefit of GC- and LC-MS based techniques is their ability to analyze complex mixtures of samples through chromatographic separations. GC–MS based techniques commonly utilize libraries to compare mass spectra of compounds detected samples to reference spectra of pure drugs. LC-MS applications for drug checking typically utilize reference standards to both qualify and quantify amounts of illicit drugs present in primarily biological matrices. Recent work has highlighted the use of paper spray mass spectrometry (PS-MS) where illicit drugs are applied to paper, solvent is added, and the paper is directly analyzed [24] In 2021, a pilot study showed the use of PS-MS on-site at a supervised consumption site in Vancouver, British Columbia, Canada and demonstrated PS-MS can provide immediate information on drug content, while maintaining sensitivity and specificity [25].

Though, the key barrier to use for any GC- and LC-MS method is the need for expensive and complex instrumentation that must be operated by trained individuals. At drug checking services MS techniques are most commonly employed as offsite, secondary analysis tools for samples that undergo primary analysis using rapid sampling technique(s) at the point of care. Despite the benefits associated with MS methods, liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for the analysis of illicit drugs in drug checking protocols are limited. The limitations for LC-MS/MS analysis as part of a drug checking protocol are the 1) a lack of developed methods for testing drugs themselves (rather than biological samples), 2) the need for access to a laboratory for sample preparation and analysis, and 3) the need for solid illicit drug samples to be collected and sent to laboratories, which is complicated by regulatory aspects. Here, we seek to illustrate the feasibility of LC-MS/MS as a secondary analysis tool for drug checking services. To do so, we are interested in 1) developing an LC-MS/MS method for sensitive and selective analysis of a variety of illicit drugs, 2) generating a method for easy sample collection and shipment from the drug checking site to the laboratory, and 3) comparing the results generated by point-of-care tools to those generated by the developed LC-MS/MS method.

Materials and methods

Reagents, chemicals, and supplies

Individual standards of all 22 analytes were purchased as Cerilliant 1 mg/mL certified reference standards in either 100 % methanol or acetonitrile from Sigma-Aldrich (St. Louis, MO) and were transferred to and stored in 1.5 mL microcentrifuge tubes. Formic acid (98 %) was purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade methanol was purchased from Fisher Scientific (Waltham, MA). LC-MS grade acetonitrile was purchased from VWR (Radnor, PA). 18 MΩ water was used for all standard, sample, and mobile phase preparations. Rapid Response Fentanyl Test Strips (FTS) at a 20 ng/mL cutoff concentration (Lot No. DOA2204278). and Rapid Response Benzodiazepine Test Strips (BTS) at a 300 ng/mL cutoff concentration (Lot No. DOA2204108) were purchased from BTNX (Pickering, ON, CA) and were run and analyzed per manufacturer recommendations. 2 mL polypropylene HPLC vials and polypropylene caps (part No. 5191–8151 and 5191–8150) were purchased from Agilent Technologies (Santa Clara, CA).

Sample collection and storage

Sample collection and the study protocol were approved through Institutional Review Boards at both the University of Notre Dame (20–07-6117, ruled exempt July 13th, 2020) and University of Illinois Chicago (2020–1047). Participants and samples were collected in the Fall of 2021 through early Spring of 2022 in the Chicago neighborhoods of Austin and Humboldt Park where the rate of opioid-related overdoses are among the highest in Cook County [26,27]. Participants were recruited from among those seeking syringe exchange and health care services at one of two street outreach facilities located within the two Chicago neighborhoods. After agreeing to participate, written informed consent was obtained, and a brief online interview on drug use, overdose history, and attitudes toward drug checking services was administered by a research assistant (RA). All participants were paid $25 each for participation in the interview and sample collection. Following the interview, the RA then asked the participant to provide a sample of their street drug. Drugs collected were in powder form, and were applied to the card by swiping a small amount of the drug using a coffee stir stick on a paper test card that can be used for rapid screening of illicit drug samples [29]. The results of rapid screening with the paper test card are not reported here as these data were used as part of neural net development and fall outside of the scope of this paper. The design of the test card (Figure S1) allowed for the provided drug sample to be deposited in a small, wax-enclosed area, protecting it from the stored reagents on the card and from the water used to run the card.

Each sample was given a unique sample ID and card number that were linked to each participant's study ID assigned during the interview. After the cards were used for rapid screening, an image of each card was taken, following which the card was air dried before being placed in an individual Ziploc bag with a small silica desiccant pad to retard moisture and prevent mold developing during the shipping process. Samples were then shipped using express mail from the Chicago collection sites to Notre Dame, IN. Upon receipt of the cards a second image was taken, and the sample ID, card number, and receipt date were recorded. Images of the card taken during sample collection and upon receipt at Notre Dame were then cross-checked to ensure all sample IDs and card numbers matched. After initial receipt, all cards were stored in their original Ziploc bags at 4 °C until extraction. After extraction, the cards were continually stored at 4 °C.

Sample extraction and preparation

For extraction, 15 mL centrifuge tubes were labeled with the unique sample IDs. Sample cards were removed from their bags and placed on a fresh paper towel. An X-acto knife was used to cut the deposition region of the card out from the rest of the paper card. The cut deposition region was then transferred to the labeled centrifuge tube using tweezers. After each card both the X-acto knife and the tweezers were rinsed with acetone and the paper towel was disposed. To get a measure of any potential carry-over between samples, a piece of card with no drug deposited on it was prepared in the same manner after every 5 samples to generate a method blank. In total, 30 method blanks were generated.

After transferring each sample to the centrifuge tubes 5 mL of the extraction solvent (90 % H2O, 10 % MeOH) was added. Samples were briefly vortexed before being sonicated for 30 min. After sonication the samples were stored, with the paper deposition region remaining in the tube, at 4 °C until preparation for LC-MS/MS on the day of instrumental analysis. Samples were prepared into labeled 2 mL polypropylene HPLC vials. 995 uL of the diluent (90:10 mobile phase A to mobile phase B) was added to the vial. Next, 5 uL of the sample was transferred to the vial. The vial was then sealed with a polypropylene cap and vortexed before analysis.

Lateral flow immunoassay analysis

Rapid Response Fentanyl Test Strips (FTS) at a 20 ng/mL cutoff concentration were used to measure for the presence of fentanyl in each sample. Samples were prepared for FTS analysis at three dilutions (neat/no dilution, 1:5, and 1:25). The 1:5 and 1:25 dilutions were diluted with 100 % H_2O. FTS were run according to manufacturer instructions. Images of each FTS were collected approximately 5 min after samples were run. Visual inspection of saved images was used to determine the presence or absence of fentanyl in each dilution. All samples and method blanks were analyzed on FTS at three dilutions in a total of 4 batches. To ensure accuracy of the FTS three dilutions of both a positive control (fentanyl present) and a negative control (no fentanyl present) were screened on FTS at the start of each batch. Positive control solutions were prepared from a 1 mg/mL LC-MS/MS standard of fentanyl prepared at 2000, 1000, and 500 ng/mL in MQ water. The 2000 ng/mL solution was prepared from the stock 1 mg/mL solution and the remaining solutions were prepared via serial dilution.

A set of positive controls prepared at concentrations ranging from 10 to 500 ng/mL in both 100 % H_2O and in the extraction solvent (90 % H_2O, 10 % MeOH) to determine the lowest concentrations that would produce a positive result on the FTS. The lowest concentration that provided a positive in both 100 % H_2O and in the extraction solvent was 100 ng/mL, higher than the listed 20 ng/mL cutoff. Neither solution produced a positive result below a 100 ng/mL concentration. Based on these results, we considered the true cutoff concentration to be 100 ng/mL and used this value when comparing LC-MS/MS and FTS results. This 100 ng/mL aligns with a previous study that found the limit of detection to be 100 ng/mL for FTS used in drug checking purposes [30].

Rapid Response Benzodiazepine Test Strips (BTS) at a 300 ng/mL cutoff concentration were used to measure for the presence of some benzodiazepines in each sample. Sample extracts were analyzed neat, without additional dilution. Images of each BTS were collected approximately 5 min after samples were run. These saved images were used to determine the presence or absence of benzodiazepines in each sample. All samples method blanks were analyzed on BTS at in a total of 2 batches. To ensure accuracy of the BTS a positive control (300 ng/mL alprazolam in 100 % H_2O) and a negative control (no benzodiazepine present) were screened on BTS at the start of each batch.

LC-MS/MS analysis

The instrument used to analyze all samples was an Agilent 1290 Infinity II adapted with a PFC conversion kit and coupled to an Agilent 6470B triple quadrupole MS/MS. For all 22 analytes ion optimization experiments were needed to determine the exact ions (precursor and products) as well as the optimal parameters (fragmentor voltage and collision energy) that are required to produce and fragment the ions. All standards were prepared from their stock 1 mg/mL solutions at a concentration of 1000 ng/mL in 90:10 water to methanol for ion optimization experiments. A total of 4 product ions and their unique collision energies were identified for each analyte. Following ion optimization, all standards were combined into a mixed stock of 50 ng/mL of each analyte for chromatography testing. The parameters for liquid chromatography were adapted from an Agilent Technologies application note on a method development of a LC-MS/MS analysis of illicit drugs in urine [31]. The determined retention time of each compound was used to determine the delta retention window which is a segment of time that represents ± 20 % of the width of the peak. The retention time and the delta retention time were used to convert the MRM transitions into a dynamic MRM (dMRM) method. All analytes were optimized and analyzed in positive mode electrospray ionization. The ion source parameters were optimized following the finalization of the chromatography with 100 % of the flow from the LC sent to the MS/MS (i.e. no splitter was used).

Method validation (linearity, precision & accuracy, LoD/LoQ) and data analysis

A detailed description of the procedures used for method validation is given in the Supporting Information. Briefly, a stock solution containing all 22 analytes was prepared at a concentration of 100 ng/mL of each analyte in the initial chromatographic conditions (90:10 mobile phase A to mobile phase B). 10 dilutions of this stock were then made at a final concentration of each analyte from 0.10 to 100 ng/mL in the initial chromatographic conditions and 1000 uL of each standard was transferred to a polypropylene HPLC vial. These vials were then used to perform each step of method validation, including an assessment of linearity, precision, accuracy, and estimations of the limit of detection (LoD) and limit of quantification (LoQ). A detailed described of the data analysis used for both method validation injections and sample injections is given in the Supporting Information. Briefly, LoD and LoQ were estimated using a previously-described process that utilizes replicate injections of blanks and low-concentration standards to first estimate a limit of blank, followed by LoD and LoQ [32]. Peak identification, integration, and analysis for all samples and standards was performed on Agilent MassHunter Quantitative Analysis for QQQ software Version 10.1.

QA/QC

Alongside the samples, a set of standards and blanks were prepared to perform QA/QC. Continuing calibration checks at a mid-range calibration point (1 ng/mL) were prepared to ensure instrumental performance was within the expected tolerances. Double blanks of the diluent (90:10 mobile phase A to mobile phase B) were prepared to measure any background levels of analyte present in the solvents or tools used during sample preparation. Each instrumental analysis batch began with a double blank injection, followed by a continuing calibration check to ensure the retention times & peak areas of each analyte were as expected. During instrumental analysis, a double blank was injected every 5 samples. A continuing calibration check was injected every 10 samples and was immediately proceeded and succeeded by a double blank to prevent carryover from the standard to samples. Additionally, every 10th sample was injected in triplicate to get a measure of intraday injection variability for the samples themselves.

Continuing calibration checks were found to have precision values within a tolerance of ± 20 % (RSD values ranged from 1 to 8 %) and accuracy values within a tolerance of ± 20 % (absolute percent difference values ranged from 0 to 17 %) except for ecgonine which had a percent difference of −29 %. For samples that were injected in triplicate during instrumental analysis to measure intraday injection variability in samples the RSD of measured concentrations ranged from 0.14 to 11.8 % (median of 1.45 %). Additionally, all replicate injections showed matched detection of analytes (i.e., all three replicates had exact overlap of analytes that were classified as non-detect, non-quantifiable, or quantifiable).

To get a measure of both interday & intraday extraction variability, three samples from cards that contained large amounts of deposited drug were extracted on two separate days. The first extraction was performed individually followed by a second day of extraction where the card was extracted in triplicate with small, less than 10 mg, amounts of sample. The exact input mass for each sample was not controlled for.

For the three samples that were prepared to measure intraday & interday extraction variability the RSD of measured concentrations of quantifiable analytes (fentanyl and diphenhydramine) ranged from 36 to 64 % (intraday) and from 32 to 53 % (interday). Importantly, all replicate samples showed matched detection of analytes (i.e., all three

replicates had exact overlap of analytes that were classified as non-detect, non-quantifiable, or quantifiable). The high variability seen in the intraday data is likely due to the lack of control or knowledge of input sample mass, leading to differences in the reported concentration of the solution.

Extraction efficiency, sample stability

Experimental protocols to measure extraction efficiency and the stability of standards and samples is given in the Supporting Information. Briefly, known amounts of 5 illicit drugs were added to paper cards as powdered solids. The cards were weighed before and after addition of the drug to determine the mass of applied solid before the card was extracted and analyzed. The concentration of drug measured was compared to the theoretical concentration using the input mass of solid drug to determine the extraction efficiency of applied sample from the paper test card using the developed extraction protocol. To measure any potential change in analyte concentrations during storage a subset of samples from batch 3 and batch 4 were analyzed on two separate occasions to measure analyte concentration changes.

Results & discussion

Method development

In addition to the precursor ion and fragmentor voltage, a total of 4 product ions and their unique collision energies were determined for each analyte. Of the 4 product ions the ion generated in the greatest abundance was used as the quantifying ion with the remaining 3 ions used as qualifying ions. For all 22 analytes their precusor and product ions, fragmentor voltage, collision energies, and cell accelerator voltages are given in Table 1. The retention time and the delta retention time of each compound used to generate the dMRM method is given in Table 1. The developed chromatographic parameters gave a total run time (including post-time) of 8.1 min, as shown in Table 2. The gradient allows all analytes to be eluted between 0.6 and 4.9 min as shown in the typical chromatograph in Figure S2. The ion source parameters used are also shown in Table 2.

Linearity, precision & accuracy, LoD and LoQ

The 10 prepared solutions ranging from 0.10 to 100 ng/mL of each analyte were injected to roughly determine the likely dynamic range for each analyte. Using this preliminary data, the concentration range of 0.10–10 ng/mL was determined as a suitable concentration range for most analytes. 6 concentrations (0.1, 0.25, 0.5, 0.75, 1, and 10 ng/mL) were injected to generate a calibration curve. The average measured peak area across the replicates of each concentration were used to examine a plot of concentration versus measured peak area. As demonstrated in Table S1 the response between concentration and measured peak area for all analytes was best described by a linear curve fit with no weighting.

Seven replicate injections of a mid-range calibration concentration (1 ng/mL) were used to measure precision & accuracy. Precision was measured by determining the RSD of the average measured peak area across these replicate injections. For all analytes the RSD values ranged from 0.8 to 9.8 % (median of 4.0 %). The same 7 replicate injections were used to determine accuracy through the percent difference of this measured concentration from the true concentration. The absolute percent difference values ranged from 0.04 to 21.7 % (median of 2.2 %). The results of precision & accuracy for each compound are given in Table S2. Interday precision values for all analytes gave RSD values ranging from 1 to 14 % (median of 3.6 %) and interday accuracy values gave absolute percent difference values ranging from 0.02 to 12.0 % (median of 2.6 %).

The LoD and LoQ for each analyte were determined as described above and in the Supporting Information. The LoD for all analytes ranged from 0.003 to 0.064 ng/mL. Table S3 gives the calculated LoD and LoQ for all analytes.

Standard and sample stability

Of the subset of 5 samples analyzed both 1 and 42 days after their extraction the same analytes were measured on both days of analysis (diphenhydramine, fentanyl, heroin, and 6-acetylmorphine). For all analytes, concentrations that exceeded the maximal 10 ng/mL value on Day 1 remained above 10 ng/mL on Day 42. For diphenhydramine and fentanyl, the percent difference between concentrations measured on Day 1 and Day 42 ranged from −12.6 % to 15.5 %. This relatively small difference in concentrations demonstrated that diphenhydramine and fentanyl concentrations were relatively unchanged during the elapsed storage period. For heroin and 6-acetylmorphine, the percent difference in concentrations measured on Day 1 and Day 42 ranged from −79.3 % to −16.4 %. The greater decrease seen here for heroin and 6-acetylmorphine affirms the observation of heroin degradation to its metabolite 6-acetylmorphine, followed by further degradation to morphine, seen in pure standards.

Extraction efficiency

The measurement of extraction efficiency of pure diphenhydramine and pure fentanyl citrate, plus cocaine, heroin, and methamphetamine (street drugs, purity unknown) was completed as described in the SI. The average recovery of each drug from these cards is given in Table S4. For the two drugs where the starting purity is known the extraction recoveries were high, 103 ± 12 % and 84 ± 24 % for diphenhydramine and fentanyl respectively, demonstrating that a significant amount of the applied mass is recovered during sample extraction with relatively low loss during additional sample prep steps. For the three analytes that have an unknown purity, the recoveries were lower, ranging from 64 to 78 %, though still within an acceptable range. For each drug the recovery of that drug when prepared in neat solution was within the standard deviation of the average recovery off the card, signaling no major losses due to the application of drug onto the paper test card.

Collected samples

A total of 138 cards were received at Notre Dame. Of the 138 cards, nine cards were excluded from extraction and analysis due to mold on the paper test card that occurred during collection and shipping. It was determined that these cards had insufficient drying time before being placed in their Ziploc bags, which promoted the mold growth. An additional five cards were excluded from future extraction and analysis due to insufficient deposition of drug in the deposition region of the card, where no visible solid was present in the deposition region. For most samples, the estimated mass collected of solid drug is estimated at 1–10 mg, though exact input mass was not recorded for samples. After these excluded samples, a total of 124 samples were included for analysis. For the 124 samples, a total of 30 method blanks were generated during preparation.

For all samples and blanks the number of days elapsed between extraction and instrumental analysis varied depending on batch number. Batch 1 (n = 56) were extracted and stored at 4 °C until sample preparation and instrumental analysis 155 days later. Batch 2 (n = 31) were extracted and stored at 4 °C until sample preparation and instrumental analysis 100 days later. Batch 3 (n = 20) and batch 4 (n = 17) were extracted and stored at 4 °C until sample preparation and instrumental analysis 1 day later. No systematic differences were observed in the analytical results produced from each batch, excluding the degradation of heroin and cocaine to their respective metabolites 6-monoacetyl mor-

Table 1
dMRM parameters from ion and chromatographic optimization experiments.

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage
6 acetyl morphine	328.2	**211.0**	1.39	0.72	114	28	4
		193.0				32	
		165.0				40	
		58.3				32	
Alpha hydroxy alprazolam	325.1	**297.1**	4.10	0.83	164	28	4
		279.1				28	
		243.1				40	
		216.1				40	
Alprazolam	309.1	**281.1**	4.25	0.85	164	28	4
		274.1				28	
		205.0				40	
		165.0				36	
Amphetamine	136.1	**119.1**	1.53	0.99	76	8	4
		91.1				20	
		77.2				40	
		65.2				40	
Benzoylecgonine	290.1	**168.0**	2.77	1.25	136	20	4
		105.1				36	
		82.2				36	
		77.1				40	
Cocaine	304.2	**182.1**	3.31	0.80	130	20	4
		150.1				28	
		105.1				36	
		82.2				36	
Diazepam	285.1	**257.1**	4.85	0.97	118	24	4
		222.1				32	
		193.0				40	
		154.0				32	
Diphenhydramine	256.2	**167.0**	3.86	0.80	82	12	4
		166.0				40	
		165.0				40	
		152.0				40	
Ecgonine	185.1	**168.1**	0.61	0.81	112	20	4
		100.1				28	
		83.1				32	
		82.2				32	
Fentanyl	337.5	**188.1**	3.82	0.77	148	24	4
		134.1				32	
		132.1				40	
		105.1				40	
Heroin	370.2	**328.2**	3.21	0.80	174	28	4
		268.1				32	
		211.0				36	
		58.3				32	
Hydrocodone	300.2	**241.1**	1.48	1.04	116	32	4
		213.1				36	
		199.0				36	
		171.0				40	
Levamisole	205.1	**178.0**	1.34	0.99	142	24	4
		123.0				32	
		117.0				36	
		91.1				40	
Lorazepam	321.0	**303.0**	4.23	0.85	138	16	4
		275.0				24	
		229.0				32	
		163.0				40	
MDMA	194.1	**163.0**	1.81	1.38	84	12	4
		135.0				24	
		133.1				20	
		105.1				28	
Methadone	310.2	**265.1**	4.08	0.83	114	12	4
		105.1				32	
		91.1				40	
		57.2				28	
Methamphetamine	150.1	**119.1**	1.74	1.13	72	8	4
		91.1				20	
		65.2				40	
		63.2				40	

(*continued on next page*)

Table 1 (*continued*)

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage
Morphine	286.2	**181.0**	0.74	0.60	164	40	4
		165.0				40	
		58.2				32	
		201.0				28	
Naloxone	328.2	**310.1**	1.04	0.92	146	20	4
		268.1				28	
		253.1				28	
		212.0				40	
Oxycodone	316.2	**298.2**	1.31	0.85	134	20	4
		256.1				28	
		241.1				32	
		187.1				32	
Oxymorphone	302.1	**284.1**	0.76	0.74	138	20	4
		242.1				28	
		227.1				32	
		198.1				40	
Phencyclidine	244.2	**159.1**	3.78	0.83	74	12	4
		91.1				40	
		86.2				12	
		81.2				24	

Table 2
Chromatographic and ion source parameters of the developed method.

Sample solvent phase:	90/10 Mobile phase A/Mobile phase B		
Mobile phase A:	90/10 H2O/MeOH + 0.1 % Formic acid		
Mobile phase B:	100 % ACN		
Column:	Waters ACQUITY UPLC BEH C18, 100x2.1 mm, 1.7 uM		
Column temperature (°C)	40		
Injection volume (uL)	5		
Time	**% A**	**% B**	**Flow (mL/min)**
0	90	10	0.4
1	90	10	0.4
1.1	75	25	0.4
2	75	25	0.4
2.1	50	50	0.4
4	50	50	0.4
4.1	10	90	0.4
6	10	90	0.4
6.1	90	10	0.4
Post-time (min):	2		
Ion source	AJS ESI		
Polarity	Positive		
Gas temp (°C)	250		
Gas flow (L/min)	10		
Nebulizer (psi)	40		
Sheath gas temp (°C)	400		
Sheath gas flow (L/min)	10		
Capillary (V)	3400		
Nozzle voltage (V)	0		

phine and morphine, and benzoylecgonine and ecgonine. For heroin and cocaine, longer storage time showed increased concentrations of its metabolites relative to samples that had shorter storage times. Some samples with longer storage time showed detection of just the metabolites and not of the compound itself. To account for this in all samples the detection of the compound and/or its metabolites were scored as detection of the compound.

FTS and BTS results

For all samples the FTS result in the neat extract and the BTS result are given in Table 3, with the FTS results at all dilutions shown in Table S5. All positive and negative controls measured at the start of each FTS batch responded as expected. All 30 method blanks were negative across all three prepared dilutions. For all 124 samples the percent of samples that showed a positive FTS result were 86 % (n = 107) in neat extract, 64 % (n = 79) at the 1:5 dilution, and 33 % (n = 42) at the 1:25 dilution. For all samples, the lowest dilution that gave a negative result also gave a negative result at the highest dilution. These results compare to existing studies of FTS response in street drug samples, including one where 91 % of suspected-opioid samples were found to be positive on a FTS after diluting 2–3 mg of sample into water[33], and another where 80 % of all samples checked were found positive on a FTS [34].

All positive and negative controls measured at the start of each BTS batch responded as expected. Of the 124 samples screened, only one sample produced a positive BTS response. These results compare to a previous study of BTS use in street drug samples where 25.8 % of samples gave a positive BTS result, though only 51 % of samples found positive with BTS were confirmed to be positive using confirmatory analysis with gas chromatography-mass spectrometry or LC-MS [35]. The BTS used here are reported by the manufacture to screen for a total of 17 benzodiazepine compounds. Though, current reports state that some of the benzodiazepines currently found in street drug samples do not have known confirmed detection using BTS, limiting the application of BTS to few benzodiazepine compounds [35–37].

LC-MS/MS results

For all 124 samples, LC-MS/MS results showed quantifiable amounts of at least 1 of the measured analytes. Table 3 gives the quantifiable analytes identified by LC-MS/MS for each sample. Compounds with the highest quantification frequency were diphenhydramine (98 %), fentanyl (91 %), and heroin (50 %) and its metabolites 6-acetylmorphine (44 %) and morphine (34 %). Given the known stability issues of heroin in aqueous solutions anytime heroin or one of its metabolites was measured above its quantification limit in a sample, the sample is shown in Table 3 as containing heroin. The same is true of cocaine and its metabolites benzoylecgonine and ecgonine. The complete detection frequencies of all compounds both below and above the quantification limit are given in Table S6. Of the 22 compounds only 12 were found above their quantification limit in any samples whereas 17 compounds were found above their detection limit. Drugs that were detectable but not present in quantifiable amounts could be traces introduced during production, distribution, or storage of these street drugs. Similarly, these low levels of drugs could be due to heterogenous samples as 1–2 mg of sample was collected for analysis from a larger sample of purchased street drug.

The quantification frequency of compounds was also broken down based on drug class as shown in Fig. 1 and illustrates that opioids were most frequently quantified (96 %), followed by stimulants (12 %), followed by benzodiazepines (1 %), and ending with non-quantified psychoactives/hallucinogenics (0 %). Co-occurrence of stimulants with

Table 3
Results of the quantifiable analytes found by LC-MS/MS, fentanyl test strip (FTS) results in neat solution and benzodiazepine test strip (BTS) results for all samples.

Sample ID	Analytes in quantifiable amounts by LC-MS/MS	FTS (neat)	BTS (neat)
AU001	Oxymorphone, fentanyl, diphenhydramine, heroin	POS	NEG
AU003	Fentanyl, diphenhydramine, heroin	POS	NEG
AU004	Levamisole, fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
AU005	Fentanyl, diphenhydramine	POS	NEG
AU006	Fentanyl, diphenhydramine, heroin	POS	NEG
AU007	Fentanyl, diphenhydramine	NEG	NEG
AU008	Levamisole, cocaine	NEG	NEG
AU009	Fentanyl, diphenhydramine, heroin	POS	NEG
AU010	Fentanyl, diphenhydramine, heroin	POS	NEG
AU011	Fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
AU012	Fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
AU013	Fentanyl, diphenhydramine	POS	NEG
AU014	Fentanyl, diphenhydramine	POS	NEG
AU015	Fentanyl, diphenhydramine	POS	NEG
AU016	Fentanyl, diphenhydramine	POS	NEG
AU017	Fentanyl, diphenhydramine	POS	NEG
AU018	Diphenhydramine, heroin	NEG	NEG
AU019	Fentanyl, diphenhydramine, heroin	POS	NEG
AU020	Fentanyl, diphenhydramine, heroin	POS	NEG
AU021	Fentanyl, diphenhydramine, heroin	POS	NEG
AU022	Fentanyl, diphenhydramine, heroin	POS	NEG
AU023	Fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
AU024	Diphenhydramine, heroin	NEG	NEG
AU025	Fentanyl, diphenhydramine	POS	NEG
AU026a	Fentanyl, diphenhydramine, heroin	POS	NEG
AU026b	Fentanyl, diphenhydramine, heroin	POS	NEG
AU027	Fentanyl, diphenhydramine	POS	POS
AU028	Methamphetamine, diphenhydramine	NEG	NEG
AU029	Fentanyl, diphenhydramine	POS	NEG
AU030	Fentanyl, diphenhydramine	POS	NEG
AU031	Fentanyl, diphenhydramine	POS	NEG
AU032	Fentanyl, diphenhydramine, alprazolam, heroin	POS	NEG
AU033	Fentanyl, diphenhydramine, heroin	POS	NEG
AU034	Fentanyl, diphenhydramine, heroin	NEG	NEG
AU035	Fentanyl, diphenhydramine, heroin	POS	NEG
AU036	Fentanyl, diphenhydramine, heroin	POS	NEG
AU037	Fentanyl, diphenhydramine, heroin	POS	NEG
AU038	Fentanyl, diphenhydramine	POS	NEG
AU039	Fentanyl, diphenhydramine, heroin	POS	NEG
AU040	Fentanyl, diphenhydramine, heroin	POS	NEG
AU041	Fentanyl, diphenhydramine	POS	NEG
AU042	Diphenhydramine	NEG	NEG
AU043	Fentanyl, diphenhydramine, heroin	NEG	NEG
AU044	Fentanyl, diphenhydramine, heroin	POS	NEG
AU045	Fentanyl, diphenhydramine, heroin	POS	NEG
AU046	Fentanyl, diphenhydramine	POS	NEG
AU047	Fentanyl, diphenhydramine, heroin	POS	NEG
AU048	Fentanyl, diphenhydramine, heroin	POS	NEG
AU049	Fentanyl, diphenhydramine	POS	NEG
AU050	Diphenhydramine, heroin	NEG	NEG
AU051	Fentanyl, diphenhydramine	POS	NEG
AU052	Diphenhydramine, heroin	NEG	NEG
AU053	Fentanyl, diphenhydramine, heroin	NEG	NEG
AU054	Fentanyl, diphenhydramine	POS	NEG
AU055	Fentanyl, diphenhydramine, heroin	POS	NEG
AU056	Fentanyl, diphenhydramine, heroin	POS	NEG
AU057	Fentanyl, diphenhydramine, heroin	POS	NEG
AU058	Fentanyl, diphenhydramine, heroin	POS	NEG
AU059	Fentanyl, diphenhydramine, heroin	POS	NEG
AU060	Fentanyl, diphenhydramine, heroin	POS	NEG
AU061	Fentanyl, diphenhydramine	POS	NEG
AU062	Fentanyl, diphenhydramine, heroin	POS	NEG
AU063	Fentanyl, diphenhydramine, heroin	POS	NEG
AU064	Fentanyl, diphenhydramine, heroin	POS	NEG
AU065	Fentanyl, diphenhydramine, cocaine, heroin	NEG	NEG
AU066	Fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
AU067	Fentanyl, diphenhydramine	NEG	NEG
AU068	Diphenhydramine	NEG	NEG
AU069	Fentanyl, diphenhydramine, cocaine	POS	NEG
AU070	Fentanyl, diphenhydramine, heroin	POS	NEG
AU071	Fentanyl, diphenhydramine	POS	NEG
AU072	Fentanyl, diphenhydramine, heroin	POS	NEG
AU073	Fentanyl, diphenhydramine	POS	NEG
AU074	Methamphetamine, fentanyl, diphenhydramine	POS	NEG
AU075	Fentanyl, diphenhydramine, heroin	POS	NEG
AU076	Fentanyl, diphenhydramine	NEG	NEG
AU077	Fentanyl, diphenhydramine, heroin	POS	NEG
AU078	Fentanyl, diphenhydramine	POS	NEG
HP001	Fentanyl, diphenhydramine, heroin	POS	NEG
HP002	Fentanyl, diphenhydramine	POS	NEG
HP003	Fentanyl, diphenhydramine	POS	NEG
HP004	Fentanyl, diphenhydramine	POS	NEG
HP005	Oxymorphone, fentanyl, diphenhydramine, heroin	POS	NEG
HP006	Fentanyl, diphenhydramine	POS	NEG
HP010	Fentanyl, diphenhydramine	POS	NEG
HP015	Fentanyl, diphenhydramine, heroin	POS	NEG
HP020	Fentanyl, diphenhydramine	POS	NEG
HP021	Diphenhydramine, heroin	NEG	NEG
HP022	Fentanyl, diphenhydramine	POS	NEG
HP023	Fentanyl, diphenhydramine, heroin	POS	NEG
HP024	Levamisole, fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
HP025	Fentanyl, diphenhydramine, heroin	POS	NEG
HP026	Fentanyl, diphenhydramine	POS	NEG
HP027	Fentanyl, diphenhydramine, heroin	POS	NEG
HP028	Fentanyl, diphenhydramine, heroin	POS	NEG
HP029	Fentanyl, diphenhydramine	POS	NEG
HP030	Fentanyl, diphenhydramine, heroin	POS	NEG
HP031	Fentanyl, diphenhydramine, heroin	POS	NEG
HP032	Fentanyl, diphenhydramine	POS	NEG
HP033	Fentanyl, diphenhydramine, heroin	POS	NEG
HP034	Fentanyl, diphenhydramine, heroin	POS	NEG
HP035	Fentanyl, diphenhydramine, heroin	POS	NEG
HP036	Methamphetamine, fentanyl, diphenhydramine, heroin	POS	NEG
HP037a	Fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
HP037b	Fentanyl, diphenhydramine, heroin	POS	NEG
HP038	Fentanyl, diphenhydramine, heroin	POS	NEG
HP039	Fentanyl, diphenhydramine	POS	NEG
HP040	Fentanyl, diphenhydramine	POS	NEG
HP041	Fentanyl, diphenhydramine, heroin	POS	NEG
HP042	Fentanyl, diphenhydramine	POS	NEG
HP043	Fentanyl, diphenhydramine, heroin	POS	NEG
HP044	Fentanyl, diphenhydramine	POS	NEG
HP045	Fentanyl, diphenhydramine	POS	NEG
HP046	Fentanyl, diphenhydramine, heroin	POS	NEG
HP047	Fentanyl, diphenhydramine, heroin	POS	NEG
HP048	Fentanyl, diphenhydramine	POS	NEG
HP049	Fentanyl, diphenhydramine, cocaine, heroin	POS	NEG
HP050	Fentanyl, diphenhydramine, heroin	POS	NEG
HP051	Fentanyl, diphenhydramine, heroin	POS	NEG
HP052	Levamisole, cocaine	NEG	NEG
HP053	Fentanyl, diphenhydramine	POS	NEG
HP054	Fentanyl, diphenhydramine, heroin	POS	NEG
HP056	Fentanyl, diphenhydramine	POS	NEG
HP057	Fentanyl, diphenhydramine, heroin	POS	NEG

opioids occurred in 80 % (n = 12) of the samples where stimulants were quantified. For the one sample where a benzodiazepine was quantified, it co-occurred with opioids.

A breakdown of the fentanyl and heroin co-occurrences is given in Fig. 1 and highlights that fentanyl was frequently measured both alone (37 %) and with heroin (58 %), with heroin being measured alone in just 5 % of samples. These results not only highlight that a significant portion of the drug market in these samples collected from Chicago, IL contain fentanyl, but also that fentanyl is being used both with and

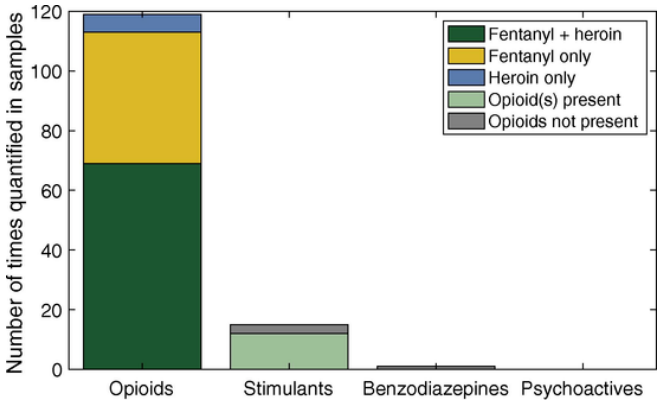

Fig. 1. Breakdown of the compounds measured in quantifiable amounts in all (n = 124) samples based on drug class. Opioids are further broken down by the presence of fentanyl and/or heroin, with other drug classes being broken down by the presence or absence of co-occurring opioids.

without heroin present. The rates of heroin and fentanyl presence in these samples aligns with the most recent data available for Chicago's opioid overdoses, where 86 % of recent opioid-related deaths involved fentanyl with 40 % being due to fentanyl alone [26,27].

Comparisons of these data to the 2019 National Drug Threat Assessment data available from the United States Drug Enforcement Agency showed that fentanyl was mixed with heroin in 37 % of samples analyzed from seizures. The same report estimates that most mixing of heroin and fentanyl is likely done at the "regional and retail" levels rather than from international organizations [38]. This estimate may explain the higher proportion of heroin and fentanyl co-occurrence (58 %) and overall low detection of heroin alone (5 %) seen here.

The cutting agent diphenhydramine was quantified in 98 % of samples whereas the cutting agent levamisole was only quantified in 3 % of the samples. Levamisole always co-occurred with cocaine, in line with previous reports from the United States Drug Enforcement Agency that described levamisole as a common cutting agent for cocaine, with 87 % of cocaine seizures containing levamisole [40].

A complete table containing each sample and the concentrations of analyte measured is given in Table S7. As the input mass of each sample during extraction is not known, the concentrations reported here are relative to the calibration curve only and are not relative to an input sample mass. For the two most commonly quantified analytes, diphenhydramine and fentanyl, their measured concentrations regularly exceeded the highest point of the calibration curve and have been reported instead as this maximal 10 ng/mL value. A subset of 5 samples where diphenhydramine and fentanyl concentrations were high were further diluted (1 in 1000) to attempt to determine their actual concentrations. Results for this subset of further diluted samples showed that only one sample had a fentanyl or diphenhydramine concentration that fell below the maximal 10 ng/mL value. In these samples, the concentration of both fentanyl and diphenhydramine in extracted solution before dilution exceed 10,000 ng/mL.

Comparison of FTS/BTS and LC-MS/MS

The comparison of FTS and LC-MS/MS results was done by using the concentration estimated by LC-MS/MS to determine if it could be detected by the FTS given the cutoff concentration. While the manufacturer reports a cutoff concentration of 20 ng/mL of fentanyl, our results indicated that 100 ng/mL of fentanyl was needed to provide a positive response on the FTS. When using 100 ng/mL as a cutoff, the FTS results for the neat extracts showed 95 % accuracy when compared to LC-MS/MS. Of the 5 % of samples that did not match all were false negatives, where the concentration of fentanyl measured by LC-MS/MS was sufficiently high to provide a positive result on FTS but was found to be negative on the FTS. False negative rates on FTS are less described than false positives, but one previous study has shown a false negative rate of 3.7 % on FTS, similar to the 5 % observed here [30]. All samples that produced a false negative at any dilution were re-run on new FTS and their results remained unchanged.

No occurrences of false positives were observed. False positives can occur on FTS when other compounds (methamphetamine and MDMA among others) are present in samples above certain concentrations [30,42]. Based on early experiments of extraction efficiency and recovered concentrations, we determined the initial extract volume of 5 mL would be sufficient to dilute any concentrations of interfering compounds below the level at which they interfere. This approach, coupled with the low occurrences of such interfering compounds in the LC-MS/MS data, prevented the occurrence of false positives in these FTS results.

Figure S3 displays the relationship between estimated concentration from LC-MS/MS and the FTS results. FTS run at the additional dilutions of extracts showed the expected results with 85 % accuracy and 100 % accuracy for the 1:5 and 1:25 dilutions respectively. The 15 % at the 1:5 dilution are again due to the occurrence of false negatives with again no false positives being observed. These results show that the concentrations at which false negatives occurred went all the way to the maximal LC-MS/MS concentration measured 2000 ng/mL. Most false negatives occurred below pre-dilution concentrations of 400 ng/mL. These results of samples also seem to affirm our measured cutoff-concentration of 100 ng/mL for these FTS. In the 114 cases where samples concentrations fell between the reported 20 ng/mL and our measured 100 ng/mL, only 41 % had positive FTS.

Based on these results, the use of FTS by diluting approximately 1–2 mg of sample into 5 mL shows a high-level of accuracy, with a false negative rate of just 5 %. Additional dilutions can be useful in determining the relative concentration of fentanyl present in a sample, should such information be of interest.

A comparison of the results from BTS to LC-MS/MS could not be made as all samples but one tested negative on BTS. For the one sample that showed a positive BTS, none of the four benzodiazepines included in this LC-MS/MS method were quantified in that sample. From LC-MS/MS one sample showed quantifiable amounts of alprazolam, though at a concentration below the manufacturer's cutoff concentration for alprazolam on the BTS. The BTS for this sample was negative. The number of benzodiazepines included in this LC-MS/MS method (n = 3 plus one metabolite) and validated for the BTS (n = 17) are low. It is likely that benzodiazepine detections are underreported here. Previous research has highlighted the large number of benzodiazepines potentially present in illicit street drug samples nationally and within Chicago, IL [26,27,35,43].

Conclusions

The developed method and its application to street drug samples highlights a new approach for drug checking that incorporates LC-MS/MS as a useful secondary analysis tool. The paper test card-based sampling approach allows for a small amount of drug (1–2 mg) to be collected, similar to the amounts of drug needed for existing drug checking tools, allowing for easy integration of LC-MS/MS as a secondary analysis tool. The high level of sensitivity and specificity that is provided by LC-MS/MS addresses current limitations in existing tools like FTIR and immunoassay strips which can suffer from poor limits of detection and interferences from co-occurring drugs or cutting agents. A key limitation for the use of LC-MS/MS in drug checking services is the cost, both to validate a method and to run samples on a routine basis as well as access to an instrument and laboratory setting. LC-MS/MS analysis should be coupled with existing drug checking analysis tools such as immunoassay strips and FTIR among others to continue to provide rapid, inexpensive results to users at the point-of-care. The results

generated here affirm the use of immunoassay strips for fentanyl in street drug samples, with the strips showing a high level of accuracy compared to a secondary analysis tool. Data provided by LC-MS/MS can offer the ability to look at complex mixtures and simultaneously detect and quantify a variety of drugs present in samples. Future studies should work to expand the number of compounds in the method, including emerging compounds of concern, such as fentanyl analogs and xylazine among others [44]. With this existing framework, additional work should be completed to explore the applicability of other LC-MS techniques, including non-targeted analysis tools which might help screen for new and emerging drugs in street drug samples.

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Uncited references

[28,39,41].

CRediT authorship contribution statement

Heather D. Whitehead : Methodology, Validation, Formal analysis, Investigation. **Kathleen L. Hayes :** Methodology, Validation, Formal analysis, Investigation. **James A. Swartz :** Conceptualization, Funding acquisition. **Elizabeth Prete :** Investigation. **Lisa Robison-Taylor :** Project administration. **Mary Ellen Mackesy-Amiti :** Conceptualization. **Antonio D. Jimenez :** Conceptualization. **Marya Lieberman :** Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2023.100475.

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