Application of dried urine spots (DUS) for non-targeted quadrupole time-of-flight drug screening

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Abstract

The use of dried urine spots (DUS) can simplify sample handling, shipment, and storage when compared to liquid urine samples. To prepare DUS, a small amount of urine is pipetted on a filter paper card. The subsequent drying of the specimen can prevent the post-sampling formation or degradation of substances (e.g. caused by bacteria). To evaluate the potential of DUS screening, 17 authentic urine samples, containing a broad range of substances, were extracted and analyzed on a Sciex 5600 TOF instrument using a non-targeted screening and library searching approach. The screening results were compared to the analysis of the same urine sample in liquid form, using the same high resolution liquid chromatography quadrupole time-of-flight mass spectrometry method.

More than 65 different legal and illegal drugs were successfully identified within the investigated 17 urine samples using the DUS screening approach. When compared to the analysis of liquid urine, the following compounds could not be identified: 1x ecgonine methyl ester, 1x nicotine, 1x promazine, 1x 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol. Overall, 95.2% of the target substances that have been detected in liquid urine were identified correctly using the DUS approach. In conclusion, DUS screening offers a simple, cost-effective, and easier sample handling alternative to the traditional use of liquid urine and provides detection of the most important substances for forensic requirements. Furthermore, the DUS sample preparation can be fully automated (sample documentation, internal standard application, and extraction).

Keywords: Dried urine spots; non-targeted screening; quadrupole time-of-flight

Introduction

In forensic and clinical settings, one of the most commonly used matrices for the detection of licit and illicit drugs is urine. Urine is used for therapeutic adherence monitoring, anti-doping analysis, and assessing recent use of drugs (1, 2). It is often preferred over blood, since substances and their metabolites can be detected over longer periods of time. In addition, the sample collection is much more convenient compared to venipuncture, unlike blood, it does not cause any clinical risk for the patient, and no trained personnel are required. For the screening of unknown substances within a sample, liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF) and immunoassays are often used. Compared to immunoassays that typically only screen for drug groups (e.g. benzodiazepines, opioids), the mass spectrometry based screening approach has the advantage of being able to distinguish and identify particular substances. This is possible, since LC-Q-TOF combines the advantages of liquid chromatography (purification and separation of analytes) with the increased sensitivity and selectivity of Q-TOF systems. Furthermore, by using non-targeted sequential window acquisition of all theoretical fragment ions spectra (SWATH^{TM}) acquisition, the complete information of every sample is stored in the dataset and can be re-processed again at a later stage.

Although liquid urine is commonly used for drug screening, the use of dried urine in combination with microsampling strategies is becoming increasingly popular, known as dried urine spot (DUS) sampling. In order to create a DUS, about 10 µL of urine are applied onto a cotton-based card or a polymer-based tip (3, 4). Key advantages of DUS include their improved matrix stability, the device handling is odorless and, sample transportation occurs without the necessity of maintaining cold chains. This is especially valuable when performing remote sampling studies, e.g. in developing countries (5, 6). Here, we

investigate whether the application of DUS sampling followed by fully automated sample extraction is a competitive alternative to the traditionally used liquid urine samples, when performing LC-Q-TOF screening (7). For this purpose, 17 authentic urine samples, containing more than 65 licit and illicit drugs (as found during an initial screening in liquid urine) were prepared as DUS and reextracted on a fully automated autosampler with two different elution solvents. Afterwards, the extracts were reanalyzed using non-targeted LC-Q-TOF screening, and reevaluated regarding signal intensities and the amount of detected substances. To the best of our knowledge this work represents the first combination of automated DUS extraction in combination with a non-targeted LC-Q-TOF drug screening approach.

Materials and methods

Chemicals and reagents

A Milli-Q® IQ 7000 system from Millipore (Billerica, USA) was used to produce de-ionized water. Acetonitrile was purchased from Thermo Fisher Scientific (Reinach, Switzerland), formic acid 50% in water from Honeywell (Grogg Chemie, Stettlen, Switzerland), ammonium formate from Sigma-Aldrich (Buchs, Switzerland), and methanol from Carl Roth (Karlsruhe, Germany). The internal standards (ISTD) ecgonine methyl ester-D_3, tramadol-^{13}C-D_3, and Δ^9-tetrahydrocannabinol-D_3 were purchased from Cerilliant (Round Rock, TX, USA).

Preparation of samples

A total of 17 urine samples, which had already been analyzed in routine procedures at the Institute of Forensic Medicine (IRM), Bern, Switzerland, were spotted on Autocollect TFN filter paper cards (CAMAG, Muttenz, Switzerland). Four spots with 10 µL of

urine were applied per card, resulting in an average DUS diameter of about 10 mm. The DUS were sent by mail to the CAMAG DBS Laboratory (Muttenz, Switzerland) for fully automated extraction. Thereby, a DBS-MS 500 HCT autosampler (CAMAG, Switzerland) was connected to a CTC PAL fraction collector (Zwingen, Switzerland) for the fully automated DUS extraction into sample vials. The filter paper cards were extracted from the center of the spot using a 6-mm diameter extraction head, within a runtime of 2 min per sample. The extraction process is shown in Figure 1. In addition, direct coupling of the autosampler to an liquid chromatography-tandem mass spectrometry (LC-MS/MS) system is possible (8). The extraction was performed using two different approaches: DUS V1, using a mixture of 100 µL of 70/30 methanol/water for the extraction of the DUS, the samples were shipped in liquid form, and 10 µL of ISTD was added upon arrival; DUS V2, extracting the DUS with 100 µL of pure methanol, followed by evaporation to dryness in a heating block at 45 °C, under a gentle stream of nitrogen, before shipment. The choice of 70/30 methanol/water for our DUS screening approach was influenced by Duthaler et al. and Gaugler et al., who had successfully applied this mixture for fully automated DBS-LC-MS/MS methods (9-11). The use of 100% methanol as extraction solvent was based on the fact that this solvent is common for the manual dried blood spot extraction for phosphatidylethanol analysis, and might therefore work as well for DUS screening approaches (12, 13). Furthermore, all compounds which have been detected with the LC-Q-TOF system in our routine general-unknown screening of forensic cases are soluble in methanol.

The dried extracts from DUS V2 were reconstituted upon arrival at the laboratory of Forensic Medicine in Bern with 100 µL reconstitution solvent, commonly used during the routine screening procedure, consisting of deionized water, acetonitrile, formic acid (97.5:2.5:0.1; v/v/v) with 2.5 mM ammonium formate, and 10 µL of ISTD was added. The first extraction method was chosen to investigate the impact of injecting a solvent mixture

with higher organic content (70% MeOH) onto the analytical column compared to the solvent routinely used for reconstitution and injection. Furthermore, this approach would allow a direct coupling of the fully automated DBS system with the LC-Q-TOF system at a later stage. The second one was added to investigate whether extraction with pure MeOH provides an advantage for the detection of certain substances compared to the extraction with 70% MeOH. The respective liquid urine samples were analyzed using the standard routine procedure applied at the IRM Bern, by diluting 30 µL 1:10 with the reconstition solvent and adding 10 µL of internal standard before injection of 1 µL.

LC-Q-TOF analysis

The substances in liquid urine and DUS were analyzed using the same validated LC-Q-TOF method, as described elsewhere (14). In short, for the non-targeted screening, 1 µL of each prepared sample from DUS V1, DUS V2, and liquid urine was injected using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Reinach, Switzerland) coupled with a TripleTOF 5600™ mass spectrometer (Q-TOF) from Sciex (Toronto, Canada) and operated with Analyst™ TF software, version 1.7 (Sciex, Toronto, Canada). The analyses were performed in positive electrospray ionization mode. The extracts were separated on a Kinetex C8 column, 50 x 2.1 mm, 2.6 µm (Phenomenex, Basel, Switzerland). A linear gradient was used (mobile phase A: water and formic acid (100:0.1; v/v); mobile phase B: acetonitrile and formic acid (100:0.1; v/v), at a flow rate of 0.35 mL/min and a total run time of 15 minutes (0 - 1 min: 2.5% B, 1 - 8 min: 2.5% - 97.5% B (linear); 8 - 12 min: 97.5% B; 12 - 12.1 min: 97.5% - 2.5% B (linear); 12.1 - 15 min: 2.5% B). Positive detection was based on a tandem mass spectrometry library match, mass accuracy, and retention time. The data were processed with MasterView™ Version 1.1 and PeakView™ Software 2.2 (Sciex, Framingham, MA, USA). The peak identification was based on the following decision criteria: a mass accuracy < 5 ppm, an isotope ratio difference < 10%, a

retention time error < 5%, and a library hit rate > 70%. The Q-TOF was operated in SWATH mode, a data-independent acquisition method. Cycle time was 0.86 seconds and the following scan parameters were applied: Mass range of 100 to 950 Da (full scan) and 50 to 950 Da (SWATH™; windows of 35 Da), scan time of 35 msec for each scan window, and collision energy of 35 eV ± 15 eV (collision energy spread).

Results

All substances identified within the 17 urine samples using LC-Q-TOF analysis in liquid urine were also found in the DUS V1 extracts, except for four compounds (first number represents the number of undetected cases, and the second number represents the total number of cases identified with the routine LC-Q-TOF analysis): Nicotine (1/1) and promazine (1/1) with signal intensities of 43'632 and 5'021 counts per second (cps) in the routine approach, one out of five positive 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) results, which already showed very low signal intensity in the routine LC-Q-TOF analysis (731 cps, mean = 10'207 cps), and one out of three ecgonine methyl ester (EME) (179'084, mean = 446'478) results. All details about the results and signal intensities can be found in Table 1. In the DUS V2 approach, six compounds (amoxicillin (1/1), EME (1/3), quetiapine (2/2), promazine (1/1), THC-COOH (1/5)) could not be detected.

For 62.3% (n = 71) of the detected compounds, a higher signal intensity was found after the DUS V1 extraction, i.e., with 70/30 MeOH/water, than after the DUS V2 extraction with 100% MeOH followed by evaporation and subsequent addition of the reconstitution solvent, while 33.3% (n = 38) showed a higher signal intensity after the DUS V2 extraction. Examples of chromatograms of four substances are shown in Figure 2. For cocaine and trimipramine, the intensities of the DUS V1 approach are higher than those of the DUS V2 approach. For benzylecgonine and ecgonine methyl ester, the signal intensi-

ties of the DUS V1 approach are lower than those of the DUS V2 approach. The exact signal intensities can be found in Table 1. No difference in the signal intensities was detectable in 1.8% (n = 2). Three substances could not be detected after either DUS V1 or DUS V2 (EME (1/3), promazine (1/1) and THC-COOH(1/5)).

The correct retention time and the comparison of the spectra with the MS/MS library spectra are important for the correct identification of the substances. Therefore a comparison of the spectra with the spectra of the library is shown in Figure 3 using the example of metformin.

Discussion

The two DUS extraction approaches DUS V1 and DUS V2 detected 95.2% (n = 110) and 92.8% (n = 98) of the 114 positive results of the routine approach, respectively. Extraction DUS V2 was additionally performed as it was suspected that direct injection of the high organic content of the extract from DUS V1 might interfere with column chromatography. Three substances (EME (1/3), promazine (1/1) and THC-COOH (1/5)) could not be detected in either approach. In the case of THC-COOH, the non-detectability can be explained by the already low intensity (731 cps, mean = 10'207 cps) in the routine approach. This could also be an explanation for the missing EME (179'084 cps, mean = 446'478 cps). The fact that a smaller sample volume is extracted in the DUS approach reduces the overall intensity and can lead to non-detectability at low concentrations. Promazine also showed a low intensity (5'021 cps). Since promazine was present only once in the 17 urine samples, additional samples have to be analyzed to determine whether DUS is in general a suitable matrix for screening of promazine using this LC-Q-TOF method. Nicotine could not be detected with the DUS V1, but with the DUS V2 approach. On the contrary, amoxicillin (1/1) and Quetiapine (2/2) could not be detected with the DUS V2, but

with the DUS V1 approach. The additional step of evaporation and reconstitution in DUS V2, as well as chemical and physical properties of the different extraction solvents could be the reason for the difference in detectability. In general, more substances could be detected with the DUS V1 approach, which also eliminates the additional step of evaporation and reconstitution of the DUS V2 approach.

The total number of urine samples (n = 17) in this first attempt is generally low, nevertheless 114 licit and illicit drugs could be tested and a promising proof of concept study could be realized. All substances, especially those that occurred only once, should also be confirmed by further measurements of larger sample sizes. This preliminary study demonstrates the proof of the concept, that detecting unknown compounds with LC-Q-TOF can be performed with both liquid urine and DUS, making it available to a larger number of users, especially in areas with lower laboratory density.

Our study has shown that it is possible to apply DUS sampling not only for LC-MS/MS analysis, but also for LC-Q-TOF approaches (15, 16). Coupling fully automated DUS extraction with an LC-Q-TOF screening method provides the opportunity to combine the lower sample volume of DUS and its easier shipping and storage with the potential detection of a wide range of analytes and, as an archival tool, the option of retrospective data analysis. Compared to previous approaches, the method shown does not require complex sample preparation, offers a broad detection range and can be performed fully automated, which would particularly benefit laboratories with a high sample throughput (16, 17). Unlinke manual extraction procedures, the dirct elution of samples from the DUS eliminates manual DUS handling (e.g. punching) and the need for consumables such as sample tubes and pipette tips (18).

Conclusions

Based on these results we have demonstrated that with the fully automated extraction of DUS using 70/30 methanol/water, more than 95% of the positive tested compounds from the routine approach could be detected. This demonstrates that the use of DUS appears to be suitable for screening illicit and licit drugs using LC-Q-TOF in the presented workflow.

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No funding was received for the realization of this study.

Competing Interests

Marc Luginbühl and Stefan Gaugler are employees of the CAMAG foundation which focuses on providing fully automated dried blood spot analysis solutions.

Data Availability Statements

The data underlying this article are contained within the article.

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Legend to figures

FIGURE 1 Fully automated dried urine spot (DUS) extraction system. The setup consists of a DBS-MS 500 HCT autosampler (CAMAG, Switzerland) connected to a CTC PAL (Zwingen, Switzerland) fraction collector.

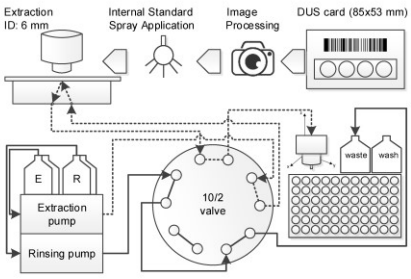


FIGURE 2 Chromatograms of ecgonine methyl ester, benzoylecgonine, cocaine (left) and trimipramine (right). In the first row the routine approach using liquid urine is shown, then the DUS V1 approach and at the bottom the DUS V2 approach.

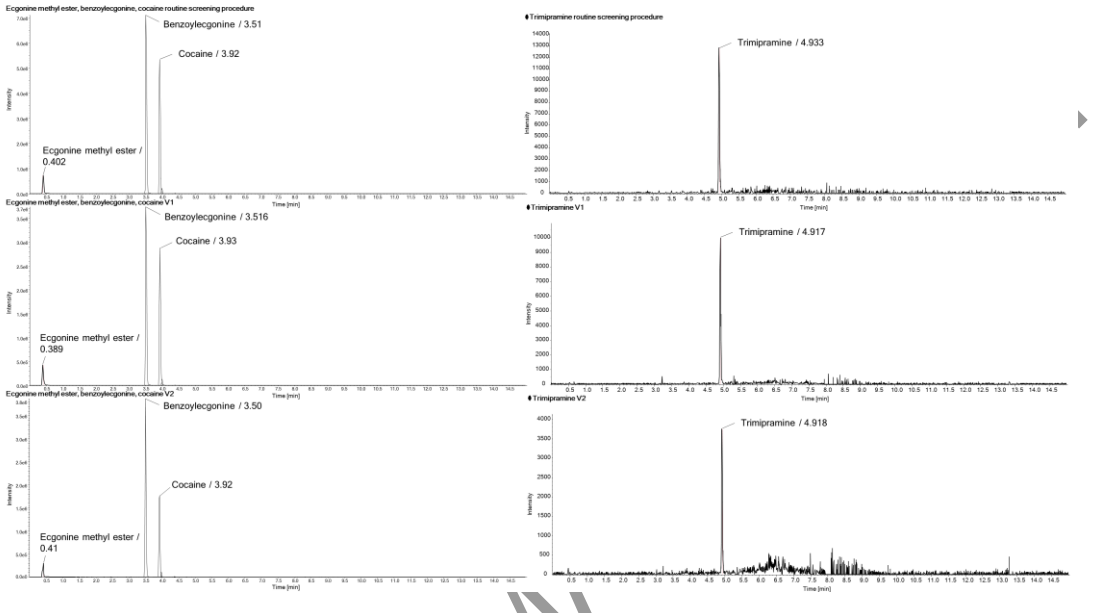
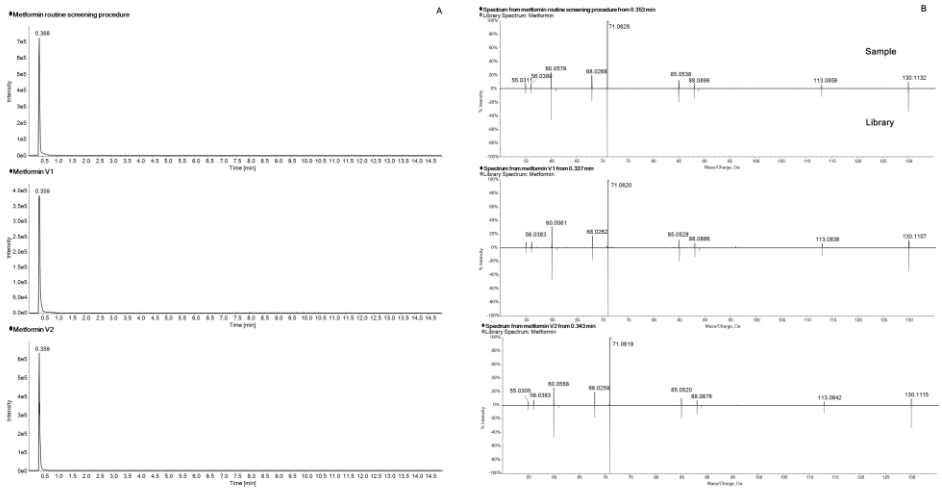


FIGURE 3 A) The example of the chromatograms of metformin shows the routine approach in the first row, then the DUS V1 approach and the DUS V2 approach at the bottom. B) The comparison between the spectra of the peaks on the left (retention times 0.353, 0.377 and 0.343) with the MS/MS library spectra is shown.



Table

TABLE 1 Details of the substances found after the three different processing techniques with the LC-Q-TOF method

Substance	Signal intensity in liquid urine with a dilution of 1:10	Signal intensity DUS V1	Signal intensity DUS V2
4-Methylaminophenazone	6'825'341	1'183'576	350'401
	4'896'741	322'938	86'110
6-Monoacetylmorphine	40'989	24'329	21'211
Alprazolam	2'779	1'911	739
	5'258	3'901	3'557
Amoxicillin	3'480'193	642'270	-
Amphetamine	1'6138	2'835	1'877
α-Hydroxymidazolam	6318	2'770	2'358
Benzoylecgonine	1'595'320	490'237	462'999
	291'445	57'977	58'071
	2'378'483	620'604	923'300
	7'105'120	3'740'525	3'839'782
Bisoprolol	145'499	29'721	40'076
Bupropion	485'053	205'494	128'747
Carbamazepine	98'567	48'301	41'702
Carbamazepine-10,11-epoxide	789'866	332'007	255'793
Citalopram	60'002	27'865	16'102

Cocaethylene	23'793	7'005	5'347
	105'474	24'004	29'959
Cocaine	70'276	27'478	18'204
	1'192'438	298'207	441'467
	5'336'534	2'879'095	2'442'396
Codeine	7'642	2'819	3'439
Codeine-6-glucuronide	14'525	2'504	4'753
Caffeine	110'327	37'097	30'499
	34'150	10'238	8'027
	102'558	35'072	36'890
	55'925	22'943	17'290
	27'145	15'319	12'282
	37'247	13'619	12'298
	21'620	12'342	9'222
	167'262	112'041	88'458
Cotinine	50'175	12'474	11'676
	26'432	7'633	7'679
Desmethylcitalopram	51'069	31'159	17'598
2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	1'397'410	422'065	297'066
Ecgonine methyl ester (EME)	179'084	-	-
	423'238	60'366	153'449
	737'111	432'337	468'958
Fentanyl	3'573	1'112	897
Gabapentin	5'796'530	3'044'522	2'725'720

Imipramine	9'432	7'481	2'953
Ketamine	1'905'289	795'500	658'085
Lacosamide	922'997	291'876	246'049
Lamotrigine	560'549	244'482	165'473
Levetiracetam	416'232	107'746	177'963
	342'504	190'477	223'464
Lidocaine	445'346	119'224	69'347
Lorazepam	799	277	292
Lorazepam glucuronide	4'365	1'375	1'208
Losartan	23'447	4'306	5'313
Metformin	724'618	385'286	633'452
Methadone	412'596	233'717	152'574
Methylphenidate	2'618'675	945'915	717'633
Metoclopramide	878'185	566'787	509'731
	718'249	446'059	429'201
Metoprolol	753'603	180'921	180'686
	549'936	130'873	156'084
Morphine	34'161	19'248	16'060
Morphine glucuronide	80'145	20'464	32'613
	46'035	4'305	5'695
N-Acetylaminoantipyrine	3'962'263	1'099'191	1'285'451
	223'903	87'026	89'293
N-Formyl-4-aminoantipyrine	1'449'248	573'944	572'936
	681'919	228'881	198'492
Nicotine	43'632	-	11'923

Norketamine	683'492	189'804	174'596
O-Desmethyltramadol (O-DSMT)	400'672	112'499	115'116
O-Desmethylvenlafaxine (ODV)	1'648'540	634'676	585'893
1-Hydroxymidazolam glucuronide	80'143	18'462	11'369
Ondansetron	292'875	85'037	78'776
Oxazepam	174'048	41'606	34'558
Paracetamol	1'506'502	819'218	925'592
	130'467	42'639	63'072
	1'008'639	283'261	462'743
	66'721	16'587	22'290
	332'451	40'971	100'745
	801'988	238'730	385'813
Pregabalin	164'457	28'515	36'258
	1'787'594	472'614	548'322
Promazine	5'021	-	-
Propranolol	19'606	14'880	8'678
Quetiapine	11'306	4'952	-
	40'051	10'649	-
Ritalinic acid	5'565'482	2'134'523	1'529'322
Sildenafil	12'239	8'630	2'915
Sitagliptin	851'346	235'695	189'205
11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-	3'795	1'367	662

COOH)			
	1'5554	13'720	6'761
	731	-	-
	2'1438	9'822	6'836
	9'517	3'427	1'391
THC-COOH-glucuronide	2'690	661	267
	14'325	9'370	3'790
	7'203	1'998	836
Theobromine, theophylline*	436'140	125'602	169'409
	96'879	16'019	29'608
	365'528	67'511	84'118
	67'398	16'831	16'717
	105'589	44'701	61'338
	312'595	160'883	93'290
	153'534	35'144	46'888
	206'845	86'820	138'715
Torasemide	66'973	18'686	15'904
	164'898	51'479	47'646
Tramadol	1'663'695	620'739	560'017
Tranexamic acid	6'218'336	2'120'771	2'964'015
Trazodone	6'994	3'697	3'220
	10'652	6'046	1'461
Trimipramine	12'845	9'978	3'764
Venlafaxine	10'755'257	383'223	354'752
Zolpidem	105'673	33'136	17'596

	9'596	4'734	2'451
Zolpidem phenyl-4-carboxylic acid	370'712	108'312	110'333

* Indistinguishable in the evaluation due to same mass and insufficient separation (one peak).