Dried Blood Spot Analysis as an Emerging Technology for Application in Forensic Toxicology

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Prepared by:
Nichole Bynum, Katherine Moore, and Megan Grabenauer
RTI International
3040 Cornwallis Road
Research Triangle Park, NC 27709-2194

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Administrative Point of Contact:
Alicia D. Brown
aliciab@rti.org
Phone: 919-541-8826
Fax: 919-541-6624

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Purpose

Forensic toxicology laboratories often receive urine, whole blood, and tissue specimens. In most cases, the most important of these three specimens is blood, because it provides information about what substances were present and the amount of substances the user was influenced by at the time of collection. Dried blood spot (DBS) analysis is well-established in newborn testing, and much work has been done to determine stability and optimal storage and extraction conditions for the analytes of interest in newborn testing; however, very little work has been done in this area for forensic applications. One benefit of DBS is the small amount of sample required for analysis, less than 100 µL compared to the 1 mL of blood required for traditional analyses. This may be especially beneficial in cases involving highly decomposed bodies, or other situations in which fluids are minimal, because the small sample volume requirements of DBS may make it possible to determine the presence of drugs when conventional toxicological analysis may be impossible or severely restricted.

The purpose of this study was to evaluate DBS analysis for its application in forensic toxicology. Specifically, to determine whether DBS could produce results comparable to traditional drug analysis and if, when combined with mass spectrometry (MS), it is sensitive enough for quantitation of the drugs of abuse typically encountered in forensic laboratories. DBS samples were evaluated using liquid-chromatography (LC) and laser diode thermal desorption (LDTD) coupled to tandem MS instrumentation for the detection of drugs relevant to forensic toxicology, including drugs of abuse, emerging designer drugs, and drugs used in drug-facilitated crimes. Our goal was to investigate the current problems encountered in DBS analysis and to evaluate the feasibility of it being implemented in forensic laboratories. This evaluation included, but was not limited to, stability, sensitivity, sample handling, extraction, and quantitation.

Project Design

The project was carried out in the following steps: 1) method development; 2) validation of drugs analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) – including on card stability; 3) analysis of authentic samples and; 4) screening validation of drugs analyzed using laser diode thermal desorption (LDTD)-MS/MS. Reference the Appendix *Materials and Methods* for details.

Step 1: Method Development

Several parameters, including card and solvent selection, spot volume, spot punch size, and internal standard (ISTD) addition methods were investigated during method development in order to establish optimal extraction conditions for 28 drugs and metabolites. The analytes were combined into two separate groups. Group 1 contained 13 analytes including opiates, antidepressants and benzodiazepines and Group 2 contained 15 analytes including amphetamines, synthetic cathinones and hallucinogens. (Table A1). The effect of hematocrit on quantitation was also investigated for DBS samples fortified with drugs from Group 2. Reference the Appendix *Method Development* for details.

Step 2: Validation of DBS Methods by LC-MS/MS

All drugs evaluated during Step 1 were validated for quantitative method analysis. Accuracy, carryover, dilution integrity, interference, linearity, limit of detection (LOD), limit of quantitation (LOQ), matrix effect, precision and stability were assessed. Reference Appendix *Validation Methods* for details.

Step 3: Analysis of Authentic Samples

Drug-positive antemortem and postmortem samples were extracted by DBS and traditional methods for comparison. Reference Appendix Extraction Methods for details.

Step 4: Validation of DBS Methods by LDTD-MS/MS

Selected drugs evaluated in Step 2 were validated for screening method analysis. Interference and LOD were assessed. Reference Appendix *Validation Methods* for details

Results

Method Development

Card and Extraction Solvent Selection

Tables 1 and 2 show the peak area ratios normalized to methanol and the DMPK-C cards. The table has been color coded for ease of interpretation as noted by the color bar below the tables. For each drug, the color green highlights the conditions (card and extraction solvent) that resulted in the highest peak areas while the color red highlights the lowest. Overall, the Whatman 903™ cards gave the best results, i.e. highest peak areas, for all drugs and was selected to use for method validation. Ethyl acetate did not work well overall and was eliminated as a potential extraction solvent early in the evaluation. Acetonitrile gave the worst recovery overall for all drugs, followed by MeOH with 0.1 % formic acid (Group 2). For the Whatman 903™ cards, there was not much difference in recovery between methanol and the acetonitrile: methanol (1:3) mixture. Methanol resulted in slightly better recovery of Group 1 drugs, while the 1:3 mixture resulted in slightly better recovery of Group 2 drugs. In order to keep the extraction method as simple as possible, methanol was chosen as the extraction solvent for both groups.

Table 1: Results of DBS card and extraction solvent evaluation for drugs in Group 1

	Methanol			1:3 Acetonitrile:Methanol			Acetonitrile		
Drugs	DMPK	903	elute	DMPK	903	elute	DMPK	903	elute
Morphine	1.00	1.83	0.25	0.84	1.03	0.18	0.04	0.05	0.06
Codeine	1.00	1.45	0.65	1.02	1.16	0.72	0.04	0.03	0.32
Oxycodone	1.00	1.15	0.67	1.12	1.13	0.78	0.09	0.11	0.36
6-acetylmorphine	1.00	1.35	0.57	0.87	0.95	0.52	0.08	0.11	0.21
Clonazepam	1.00	1.29	0.65	1.08	1.20	0.69	0.18	0.09	0.35
7-aminoclonazepam	1.00	1.16	0.49	0.97	1.10	0.56	0.11	0.12	0.25
Zolpidem	1.00	0.96	1.42	1.08	1.04	1.62	0.06	0.05	0.66
Trazodone	1.00	1.00	1.29	1.05	1.04	1.43	0.11	0.10	1.11
Citalopram	1.00	0.97	1.17	1.02	0.94	1.30	0.08	0.07	0.61
Amitriptyline	1.00	0.84	1.53	1.12	0.98	1.61	0.10	0.07	1.10
Nortriptyline	1.00	1.04	1.21	1.42	1.33	1.37	0.11	0.07	0.91
Alprazolam	1.00	1.14	0.80	1.05	1.10	0.85	0.05	0.04	0.37
Diazepam	1.00	1.21	0.90	1.07	1.12	0.97	0.07	0.07	0.73

Table 2: Results of DBS card and extraction solvent evaluation for drugs in Group 2

	Methanol			1:3 Acetonitrile:Methanol			Acetonitrile			MeOH with 0.1% Formic Acid		
Drugs	DMPK	903	elute	DMPK	903	elute	DMPK	903	elute	DMPK	903	elute
AMP	1.00	1.17	0.45	1.08	1.26	0.47	0.28	0.31	0.46	0.79	0.77	0.46
BZE	1.00	1.15	0.79	1.19	1.29	0.83	0.03	0.02	0.23	1.10	1.15	0.83
Cocaine	1.00	1.13	1.23	1.38	1.27	1.59	0.08	0.08	0.72	1.12	1.19	1.43
Ketamine	1.00	1.15	1.22	1.36	1.29	1.47	0.11	0.11	0.97	0.86	0.96	1.36
LSD	1.00	1.07	1.64	1.53	1.29	2.13	0.05	0.05	0.73	0.41	0.53	1.68
MAMP	1.00	1.13	1.06	1.33	1.32	1.21	0.24	0.26	0.99	0.78	0.76	1.12
MDA	1.00	1.17	0.40	1.09	1.26	0.44	0.18	0.20	0.33	1.07	1.14	0.44
MDEA	1.00	1.14	1.15	1.38	1.31	1.38	0.16	0.17	0.91	0.94	1.00	1.27
MDMA	1.00	1.13	0.84	1.33	1.33	0.95	0.17	0.18	0.69	0.94	1.01	0.88
MDPV	1.00	1.14	1.91	1.31	1.23	2.45	0.12	0.12	1.18	0.77	0.89	2.15
Mephedrone	1.00	1.14	1.20	1.30	1.27	1.41	0.30	0.33	1.18	0.40	0.38	1.17
Methylone	1.00	1.14	0.59	1.24	1.28	0.65	0.24	0.26	0.53	0.65	0.70	0.57
PCP	1.00	1.11	1.57	1.32	1.18	2.02	0.08	0.08	0.97	0.72	0.78	1.78
Pseudoephedrine	1.00	1.28	0.43	1.20	1.47	0.43	0.17	0.21	0.38	0.45	0.49	0.41
α-PVP	1.00	1.15	2.18	1.23	1.19	2.75	0.15	0.16	1.60	0.51	0.55	2.46

Spot Volume and Spot Punch Size

Three punch sizes (3mm, 6mm, and whole punch) were evaluated for each of two spot volumes (30 µL and 50 µL). 3mm punches proved difficult to handle in the laboratory as they were easily dropped and susceptible to static charge from the microcentrifuge tubes used for extraction. Whole spots were time consuming to excise, and did not fit in microcentrifuge tubes. They had to be extracted in much larger test tubes. 6mm punches were chosen to complete the validation because they were easier to obtain (manual punch), than whole spots (excision by hand) and could easily be submerged in the extraction solvent in microcentrifuge tubes. The reproducibility of peak areas for all drugs extracted did not vary significantly between 30 µL and 50 µL spots, with %CVs of 6.5% and 6.8%, respectively. The validation was conducted using a 30 µL blood spot volume because a 6mm punch contains a larger portion of the blood spot compared to a 50 uL spot, giving greater sensitivity, and which we felt would lead to greater long term reproducibility of the assay.

ISTD Addition Methods

Table 3 shows the reproducibility of the overall average response ratio, expressed as %CV, for each of the three ISTD addition methods tested for drugs and metabolites in Group 1. Overall, reproducibility was comparable among the three methods, ranging from 7.3- 8.1%. Although adding the ISTD into the blood prior to spotting on the card is ideal, because it allows the ISTD to compensate for the entire extraction process, it is not practical or even possible in certain situations, such as when DBS samples are stored for future quantitation of unknown drugs or arrive at the laboratory pre-spotted for testing. Therefore the method of adding the ISTD into the extraction solvent was selected. This method was selected for the extraction of drugs in Group 2 without any further evaluation.

Table 3: Results of ISTD addition method evaluation of drugs in Group 1

Method of ISTD Addition	Overall Average Response Ratio (CV%)
In Extraction Solvent	7.6
In Blood	7.3
Onto Card	8.1

Hematocrit

Blood with high hematocrit levels quantified artificially high, while blood at low hematocrit quantified artificially low for all cards, regardless of punch size. However, for the samples that were investigated during this study, there did not appear to be a large bias within the normal hematocrit range (38-54%). Figure A2 shows the average response ratios, for drugs in Group 2, normalized to 45%, the average adult hematocrit level.

Validation of quantitative analysis method by LC-MS/MS

Precision and Accuracy

The overall grand accuracy for all drugs ranged from 86.3% for MDA to 112.7% for citalopram. The between-run precision, expressed as %CV, ranged from 2.1% for ketamine to 15.1% for MDPV, while the within-run precision ranged from 4.7% for morphine to 9.6% for trazodone (Table A5).

Limit of Detection (LOD)/Limit of Quantitation (LOQ)

The LOD ranged from 0.1 ng/mL to 2 ng/mL and LOQ from 2 ng/mL to 10 ng/mL, with 6-acetylmorphine and clonazepam having the highest values for both parameters (Table A5).

Linearity

Linearity ranged from the LOQ-500 ng/mL for all drugs with the exception of clonazepam, which had a linear range from LOQ-200 ng/mL and 7-aminoclonazepam, which had a linear range from LOQ-300 ng/mL. A quadratic curve was used for the quantitation of oxycodone, because its upper limit of linearity range was 150 ng/mL. The correlation coefficients (r^2) ranged from 0.991-0.998 for all analytes (Table A5).

Matrix Effect

Matrix effect values ranged from 76% for amitriptyline to 136% for trazodone at 10 ng/mL and from 77% for nortriptyline to 121% for morphine at 50 ng/mL (Table A6). Recovery ranged from 34% for α-PVP and LSD to 65% for BZE at 10 ng/mL and from 35% for LSD to 71% for BZE at 50 ng/mL. The exceptions were 6-acetylmorphine and morphine, which had recoveries between 13-15% at both concentrations.

Dilution Integrity and Interference

No interferences were detected from ISTD, matrix or commonly encountered analytes. The overall accuracy of the diluted samples ranged from 95-109% with the exception of α-PVP, which had an accuracy of 126%.

Stability

For most of the compounds studied, the concentrations extracted from DBS cards decreased with increasing storage time for all three storage conditions. Notable exceptions were 6-acteylmorphine and morphine, which increased by more than 50% after two weeks of storage at room temperature, and LSD, which increased by more than 60% after 12 weeks of storage at both room temperature and 4˚C.

Of all the storage conditions tested DBS cards stored at 4˚C were the most stable. At 12 weeks, 20 out of the 28 compounds studied were within 20% of their baseline concentrations and therefore considered stable. Exceptions were oxycodone (79%), zolpidem (76%), amitriptyline (79%), nortriptyline (79%), clonazepam (78%), LSD (124%), mephedrone, (66%), and methylone (48%). After 20 weeks of storage at 4˚C, 18 out of 28 compounds were stable in DBS compared to 8 out of 28 that were stable in whole blood. The stability of several compounds that were the least stable in whole blood was significantly improved by storage in DBS: 6AM (4 days vs. 16 weeks), cocaine (2 weeks vs. 20 weeks), mephedrone (4 days vs. 8 weeks), and methylone (1 week vs. 8 weeks). Full stability results are summarized in the Appendix in figures A4 (Group 1 drugs) and A5 (Group 2 drugs).

Analysis of Authentic Postmortem Samples

Twenty-five postmortem samples were analyzed for BZE using DBS and traditional acetonitrile crash methods (see Figure A3). In our initial analysis BZE concentrations were approximately twice as high in the DBS samples as they were in the traditionally extracted samples. This may have been caused by the difference between drug-free blood used to make the calibrators, which was prepared from packed red blood cells, and the postmortem blood. During routine screening of the blood purchased to make the calibrators for the quantitation of the postmortem samples BZE and cocaine were found to be present. The manufacturer verbally screened the participants, from whom the blood was taken, however RTI screening of blood determined that the participants were not drug-free as they stated, therefore the blood could not be used to prepare calibration curves. Drug-free antemortem blood was quickly obtained from a

local laboratory, however it was prepared from packed red blood cells. The postmortem blood was more viscous and spread less than the blood prepared from packed red blood cells. This difference in viscosity was problematic, because the ISTD was added into the extraction solvent. One option to alleviate this problem is to prepare a curve using blood with a similar viscosity. When the calibration curve was prepared using postmortem blood instead of packed red blood cells, there was good correlation between the postmortem samples extracted by DBS and the samples extracted using traditional methods (% difference less than 20% for 19 samples). Another option to deal with samples of very different viscosities, is to add the ISTD to the blood prior to spotting.

Analysis of Authentic Antemortem Samples

Twenty-two authentic antemortem samples, many containing multiple analytes (n=33), were analyzed for alprazolam, amphetamine, benzoylecgonine, clonazepam, cocaine, codeine, diazepam, methamphetamine, morphine, PCP, trazadone, and 7-aminoclonazepam using DBS and supported liquid extraction (SLE). The % difference of the concentrations between the two extraction methods ranged from 0.1% for alprazolam to 76% for MAMP.

Validation of screening methods by LDTD-MS/MS

LOD and Interference

The LOD ranged from 2 ng/mL for zolpidem, citalopram, MDEA and cocaine to 50 ng/mL for codeine (Table A5). LOD values and interference assessment was not obtained for 6-acetylmorphine, amphetamine, α-PVP, clonazepam, morphine and trazodone due to the presence of an anomalous peak in the drug-free blood. There were no interferences present with the exception of hydrocodone, which interfered with the analysis of codeine, and mephedrone, which interfered with analysis of zolpidem.

Scholarly Products

Planned Publications

Dried Blood Spot Analysis as an Emerging Technology for Application in Forensic Toxicology
Nichole Bynum, Katherine Moore and Megan Grabenauer
Manuscript in preparation

Presentations

The Importance of Evaluating Internal Standard Addition Methods in Dried Blood Spot Analysis
Nichole Bynum, Katherine Moore and Megan Grabenauer
Presented at the Society of Forensic Toxicologist Meeting, October 18-23, 2015 Atlanta, GA

D**ried Blood Spot Analysis as an Emerging Technology for Application in Forensic Toxicology**
Nichole Bynum, Katherine Moore and Megan Grabenauer
Presented at the NIJ R&D Symposium at the American Academy of Forensic Sciences, February 22-27, 2016 Las Vegas, NV

Implication for Policy and Practice

Hundreds of thousands of controlled substances and drugs of abuse are analyzed in forensic laboratories each year and are submitted as evidence in judicial cases. Forensic laboratories are often faced with a large influx of samples requiring testing and continue to benefit from ways to modernize sample analysis, including more effective ways to test samples for drugs of abuse. The success of DBS for use in forensic laboratories not only impacts the way samples are analyzed, but also the way in which they are stored, transported, and in many instances, collected. DBS requires a small amount of sample, which is useful in cases for which there is limited sample. This impacts the judicial system by allowing for toxicological analysis of samples that may otherwise go untested. The small sample size also decreases the risk of exposure to blood-borne pathogens, making it safer for those involved in sample

collection and analysis. The potential for longer-term storage and increased stability allows samples to be re-analyzed in the event new evidence is needed years after collection.

Although DBS has been well established for use in clinical settings, it has not been applied in forensic toxicology. Our research shows that quantitative LC/MS/MS results with DBS are all within recommended guidelines from such entities as the Society of Forensic Toxicologists indicating that these results are comparable to well-established extraction methods for whole blood toxicology analyses. In addition, this research sheds light on important method development parameters that must be considered prior to validating and implementing DBS analysis in the laboratory.

Appendix

Materials and Methods

Drug standards for preparing calibrators and quality controls were purchased from Cerilliant (Round Rock, TX) and Cayman Chemical (Ann Arbor, MI). All reagents were high-performance liquid chromatography (HPLC) grade. Acetonitrile, water, methanol, dichloromethane, ethyl acetate and ammonium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Sigma Aldrich (St. Louis, MO). Ammonium formate and formic acid were purchased from Alfa Aesar (Ward Hill, MA) and Electron Microscopy Sciences (Hatfield, PA), respectively. Hydrochloric acid and sodium chloride were purchased from Aqua Solution (Deer Park, TX) and BDH (West Chester, PA), respectively. Human whole blood was purchased from BioreclamationIVT (Hicksville, NY) and Equitech Enterprises, Inc. (Kerrville, Texas). Postmortem blood and drug-free human blood prepared from packed red-blood cells was received from a local medical examiner's office.

Table A1: Grouped drugs and metabolites evaluated and validated by DBS

Drugs and Metabolites	
Group 1	**Group 2**
6-acetylmorphine	α-PVP
7-aminoclonazepam	Amphetamine
Alprazolam	Benzoylecgonine
Amitriptyline	Cocaine
Citalopram	Ketamine
Codeine	LSD
Clonazepam	MDA
Diazepam	MDEA
Morphine	MDMA
Nortriptyline	MDPV
Oxycodone	Mephedrone
Trazodone	Methamphetamine
Zolpidem	Methylone
	PCP
	Pseudoephedrine

Extraction of DBS Samples

Fortified blood (30 µL) was spotted onto the card and allowed to dry for 3 hours. A 6mm Whatman™ Uni-Core™ punch (GE Healthcare Bio-Sciences; Pittsburgh, PA) was taken from the center of the spot and placed into a microcentrifuge tube. Extraction solvent (250 µL) containing ISTD was added and the samples were sonicated for 20 minutes and centrifuged at 4000 rpm for 2 minutes. The solvent was transferred into a glass test tube, evaporated under nitrogen at 40°C and 30°C for samples containing drugs from Group 1 and Group 2, respectively. Methanol containing 0.25% hydrochloric acid (10 µL) was added to samples containing drugs from Group 2 in order to prevent loss of amphetamine. Samples were dried down at 40°C and 25°C for Group 1 and Group 2, respectively. Each Group of drugs was reconstituted in 100 µL of mobile phase.

SLE Extraction Method for Whole Blood Samples

Whole blood samples were extracted using ISOLUTE supported liquid extraction (SLE) from Biotage (Charlotte, NC). ISTD (10 µL) was added to 200 µL of calibrators and whole blood samples. Samples were vortexed and 200 µL of 1% ammonium hydroxide in water was added and vortex mixed. Samples were loaded onto the SLE cartridge (375 µL). Once all samples were loaded on the cartridges, they were allowed to sit for 5 minutes. Samples were then eluted with 2 x 1 mL of dichloromethane. Methanol containing 0.25% hydrochloric acid (10 µL) was added to samples containing drugs from Group 2. Samples were dried down at 40°C and 25°C for Group 1 and Group 2, respectively. Each Group of drugs was reconstituted in 100 µL of mobile phase.

Acetonitrile Crash Method for Whole Blood Samples

ISTD (10 µL) was added to calibrators and whole blood postmortem samples (100 µL), followed by NaCl (200 µL) and acetonitrile (500 µL). After samples were vortexed and centrifuged (5 min, 4000 rpm), the organic layer was evaporated under nitrogen to dryness (5 min, 40 °C) and reconstituted in mobile phase (95:5, 5mM ammonium formate, 0.1% formic acid: acetonitrile, 0.1% formic acid, 50 µL).

Extraction of DBS samples for LDTD analysis

Fortified blood (30 µL) was spotted onto the card and allowed to dry for 3 hours. A 6mm punch was taken from the center of the spot and placed into a microcentrifuge tube. Methanol (250 µL) was added and the samples were sonicated for 20 minutes. Methanol containing 0.25% hydrochloric acid (10 µL) was added to samples containing drugs from Group 2. Samples were dried down at 40°C and 30°C for Group 1 and Group 2, respectively. Each Group of drugs was reconstituted in 75:25 methanol:water (50 µL). Samples were spotted (5µL) onto EDTA pretreated Lazwell plates and allowed to dry at room temperature.

Card and Extraction Solvent Selection

Three card types, two chemically untreated (Whatman 903™ and Whatman FTA ™ DMPK-C), and one chemically treated (Whatman Elute™) were evaluated during this study. Based on what was promising in the literature, the following extraction solvents were simultaneously evaluated along with card type for Group 1: methanol, acetonitrile, acetonitrile: methanol (1:3), and ethyl acetate. The same solvents were evaluated for Group 2 with the exception of ethyl acetate, which was replaced by methanol containing 0.1 % formic acid. Drug-free human blood was fortified with drugs listed in Group 1 and separately with drugs listed in Group 2, at 10 ng/mL and 50 ng/mL each. The three cards were spotted with fortified blood at both concentrations, for each extraction solvent in replicates of five.

Spot Volume and Punch Size

Fortified blood was spotted at 30 µL and 50 µL at 2, 10 and 50 ng/mL in replicates of five. Based on the results of the card and extraction solvent evaluation, Whatman 903™ cards and methanol were used to evaluate spot volume, spot punch size and ISTD addition methods. Three sizes (whole spot, 3mm and 6mm diameter punches) were taken from DBS fortified at 2, 10 and 50 ng/mL and analyzed in replicates of five.

ISTD Addition Methods

In order to determine the optimal methods for ISTD addition, the following methods were evaluated using blood fortified at 2, 10 and 50 ng/mL in replicates of five: 1) adding ISTD to the blood prior to spotting; 2) adding ISTD onto the blank card and allowing it to dry prior to applying the blood spot; and 3) adding ISTD into the extraction solution.

Hematocrit

The effect of hematocrit on quantitation was evaluated by analyzing drugs from Group 2, at 15 ng/mL and 400 ng/mL fortified in blood, in replicates of five, with hematocrit levels of 25, 35, 45, 55, 65 and 75%.

LC-MS/MS Methods

Validation Methods

Samples were analyzed on an Agilent 6490 triple quadrupole (MS/MS) with an electrospray source operating in positive mode coupled to an Agilent 1290 high performance liquid chromatography system (Santa Clara, CA). The analytical method parameters and monitored ion transitions and optimized parameters are shown in Tables A2 and A3, respectively.

Table A2: LC-MS/MS Analytical Method Parameters

	Drug Group 1			**Drug Group 2**		
Analytical Column	Agilent Poroshell 120 SB-C18 (2.1 x 100 mm, 2.7 mM)					
Mobile Phase	(A) 5 mm ammonium formate with 0.1% formic acid (FA) (B) Methanol with 0.1% FA			(A) 5 mm ammonium formate with 0.1% FA (B) Acetonitrile with 0.1% FA		
Mobile Phase Gradient	**Time (min)**	**%A**	**%B**	**Time (min)**	**%A**	**%B**
	0	95	5	0	90	10
	2	85	15	1.5	85	15
	2.5	30	70	3.5	70	30
	3	30	70	3.6	10	90
	5	5	95	4.5	10	90
	6	5	95			
Flow Rate	0.4 mL/min			0.8 mL/min		
Injection Volume	10 µL					

Column Temperature	55°C	50°C

Table A3: Monitored ion transitions and optimized parameters for drugs and metabolites

Drug	Precursor Ion (m/z)	Collision Energy Voltage (V)	Product Ion 1 (m/z)	Collision Energy Voltage (V)	Product Ion 2 (m/z)
Morphine	286	70	152	44	165
Morphine-d3	289	70	152	44	
Trazodone	372	24	176	44	148
Trazodone-d6	378	28	182	44	
Zolpidem	308	36	235	56	92
Zolpidem-d6	314	44	235	56	
Oxycodone	316	20	298	36	241
Oxycodone-d3	319	20	301	36	
Nortriptyline	264	20	105	32	91
Nortriptyline-d3	267	20	91	32	
Diazepam	285	36	193	32	154
Diazepam-d5	290	40	198	32	
Codeine	300	56	165	52	152
Codeine-d3	303	70	152	52	
Clonazepam	316	28	270	44	214
Clonazepam-d4	320	28	274	44	
Citalopram	325	36	109	20	262
Citalopram-d4	331	32	109	20	
Amitriptyline	278	16	233	48	91
Amitriptyline-d3	281	16	233	48	
Alprazolam	309	28	281	52	205
Alprazolam-d5	314	28	286	52	
6-AM	328	28	211	40	165
6-AM-d3	331	32	211	40	
7-aminoclonazepam	286	32	121	29	250
7-aminoclonazepam-d4	290	32	121	29	
LSD	324	24	223	52	207
LSD-d3	327	24	226		
Cocaine	304	20	182	40	105
Cocaine-d3	307	20	185		
Benzoylecgonine	290	20	168	32	105
Benzoylecgonine-d8	298	20	171		
MDPV (coc-d3)	276	32	135	28	126
PCP	244	44	91	8	159
PCP-d5	249	40	96		
Ketamine	238	36	125	70	89

Ketamine-d4	242	32	129		
α-pvp (coc-d3)	232	28	126	28	91
MDEA	208	12	163	28	105
MDEA-d5	213	8	163		
Methylone (amp-d5)	208	16	160	28	132
MDMA	194	8	163	24	105
MDMA-d5	199	12	165		
MDA	180	8	163	44	77
MDA-d5	185	8	168		
Mephedrone (bze-d8)	178	8	160	20	145
Pseudoephedrine	166	8	148	24	115
Pseudoephedrine-d3	169	8	151		
Methamphetamine	150	24	91	8	119
Methamphetamine-d5	155	20	92		
Amphetamine	136	16	91	4	119
Amphetamine-d5	141	16	93		

Linearity

The calibration curve spanned the range of biologically relevant concentrations using non-zero calibrators for all drugs listed in Table A1. The calibration curves were established, extracted and analyzed in replicates of five (n=5 at each concentration level). Each analyte's respective stable isotope labeled compound was used as an ISTD with the exception of MDPV and α-PVP, which used COC-d3 and an ISTD; and methylone and mephedrone which used AMP-d5 and BZE-d8 as ISTDs, respectively.

Precision and Accuracy

Precision and accuracy were determined by analyzing three quality control (QC) samples at the lower, middle and upper portion of the calibration curve. Each sample was analyzed in triplicate within each linearity run over the course of five runs.

Limit of Detection (LOD)

The LOD was determined by analyzing three sources of blood matrix fortified in decreasing drug concentrations, in duplicate over three runs. The LOD was the concentration of the fortified sample that consistently yielded a signal greater than the average signal of the drug-free sample plus 3.3 times the standard deviation.

Limit of Quantitation (LOQ)

The LOQ was defined as the concentration of the lowest calibrator, which was administratively set during the establishment of the calibration curve.

Carryover

Carryover was determined by analyzing blank sample matrix immediately after a high concentration sample in each calibration curve (n=5). The highest concentration at which no analyte carryover was observed (above LOD) in the blank matrix sample was determined to be the concentration at which the method is free from carryover.

Dilution Integrity

The effect of sample dilution was evaluated by repeating precision and accuracy studies of a sample at a high drug concentration diluted at 1:10 and 1:50 ratios. Each diluted sample was analyzed in triplicate over five runs.

Interference

Ten different blank lots of drug-free blood matrix were analyzed by LC-MS/MS without addition of ISTD to evaluate interference from the matrix. Matrix was considered to interfere with an analyte if the average area of the blank samples (n=10) was greater than the LOD. In addition, five blank matrix samples containing ISTD were analyzed to demonstrate the absence of interferences originating from ISTD. A sample was considered to have interference if the average peak area of the blank + ISTD samples (n=5) was greater than the LOD. Finally, fortified matrix samples containing drugs, at 1,000 ng/mL, commonly encountered in the laboratory were evaluated to determine if they had the potential to interfere with the method's analytes. Table A4 lists the analytes that were evaluated as potential interferences. A potential interfering analyte was considered to interfere if its area was greater than the LOD of the target analyte.

Table A4: List of analytes evaluated as potential interferences

Mix 1	Mix 2	Mix 3	Mix 4
Amphetamine	Hydrocodone	Methylone	Butabarbital
Methamphetamine	Hydromorphone	Mephedrone	Butalbital
MDA	Oxycodone	MDPV	Zaleplon
MDMA	Oxymorphone	Pentedrone	Zopiclone
MDEA	Fentanyl	Pseudoephedrine	Zolpidem
PCP	Methadone	Ephedrine	Fluoxetine
LSD	Desomorphine	Phenylephrine	Triazolam
Cocaine		Phenylpropanolamine	
Benzoylecgonine			
Ketamine			

Matrix Effect

Matrix effects were evaluated using the method described by Matuszewski and colleagues (Matuszewski, 2003). Three sets of samples were created for each target analyte. As described by Matuszewski and colleagues, comparative calculations were used to evaluate the data:

ME (%) = B/A x 100
RE (%) = C/B x 100

where A, B, and C = the mean responses as represented by the area under the peaks for target and internal standard quantitative ions, ME = matrix effect, and RE = recovery efficiency. Type A samples are target analytes and ISTD in mobile phase. Type B samples are drug-free blood matrix extract post extraction spiked with target analytes and ISTD. Type C samples are drug-blood matrix spiked with ISTD and target analytes prior to extraction. The mean responses for A, B, and C were determined across these 10 blood

matrix lots. The assessment of a relative matrix effect was determined by comparing the MEs between the 10 lots. The variability (%CV) in the MEs between lots is considered to be a measure of the relative matrix effect.

Stability

Low, mid and high QC samples were used to evaluate processed stability. DBS samples of each concentration were analyzed in triplicate immediately after extraction to establish the time zero peak area ratios. All remaining vials containing stability samples were stored at room temperature on the autosampler. The remaining vials were then analyzed in triplicate at 24, 48 and 72 hours. The average peak area ratios at each time interval are compared to the time zero ratios. The analyte was considered stable until the average ratios compared to those of time zero fell outside the range of ± 20 %. For short and long-term stability studies, three sets of cards were spotted at low and high QC concentrations and stored in a plastic bag with desiccant at room temperature in a dark drawer, at 4°C in a refrigerator and at -20°C in a freezer. Samples were analyzed in triplicate up to 20 weeks (Figure A1).

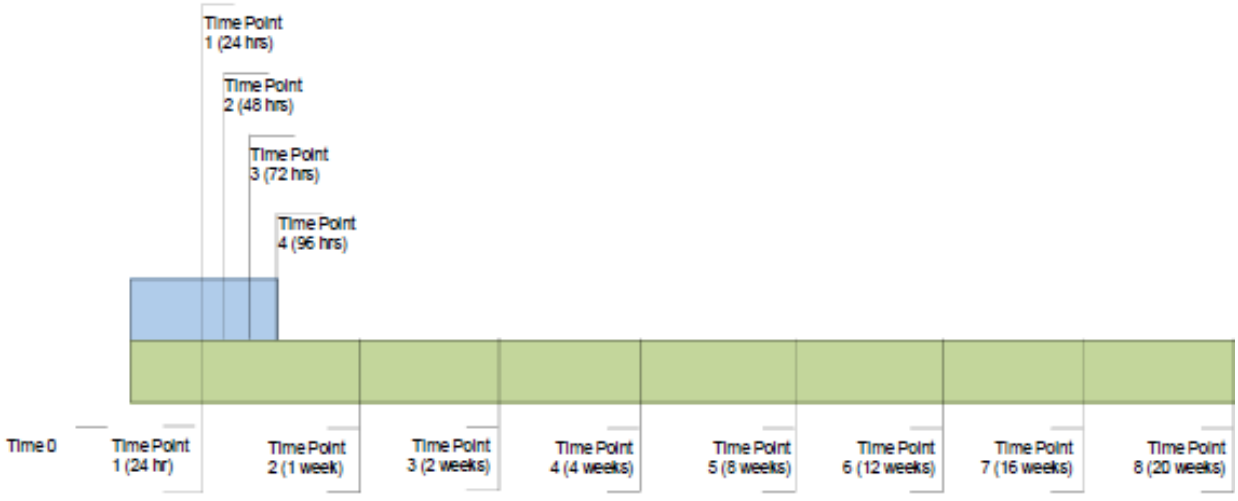

Figure A1: Timeline for short-term (blue) and long-term (green) stability studies.

Screening Validation Methods

Samples were analyzed on a model T-960 LDTD-APCI ionization interface, controlled by LazSoft 4.0 Software (Phytronix Technologies, Quebec, Canada) was installed on an ABSciex (Framingham, MA) API 4000 tandem MS controlled by Analyst Software (Version 1.4.2) (Foster City, CA). The LDTD carrier gas (compressed air) flow rate was set to 3 L/min. The laser power and timing parameters for Group 1 analytes were 0 % to 45 % in 3 sec and 45% to 0% in 0.1 sec (3-45-0). The parameters for Group 2 analytes were 0 % to 25 % in 3 sec, hold at 25% for 2 sec, and 25% to 0% in 0.1 sec (3-25-2).

Table A4: LDTD-MS/MS monitored ion transitions and optimized parameters for drugs and metabolites

	Drug	Precursor Ion (m/z)	Product Ion 1 (m/z)	CE (V)	DP (V)
Group 1	Nortriptyline	264	233	20	50
	Amitriptyline	278	233	25	50
	Diazepam	285	154	40	70
	MOR	286	201	35	70
	COD	300	215	35	90
	Zolpidem	308	235	48	50
	Alprazolam	309	281	35	50
	Clonazepam	316	270	35	50
	Citalopram	325	262	25	50
	6-AM	328	211	30	80
	Trazodone	372	148	45	40
Group 2	AMP	136	119	15	30
	MAMP	150	119	15	40
	MDA	180	163	15	40
	MDMA	194	163	15	45
	MDEA	208	163	20	40
	Methylone	208	160	25	50
	α-PVP	232	126	35	70
	PCP	244	159	20	40
	MDPV	276	126	35	70
	BZE	290	168	27	40
	COC	304	182	27	40

Validation

Table A5: Accuracy, precision, correlation coefficient (r^2), LOD and LOQ results for 28 drugs and metabolites

Drug Analytes	Overall Grand Accuracy	Between-run Precision	Within-run Precision	r^2	LOD LC-MS/MS	LOD LDTD-MS/MS	LOQ
6-Acetylmorphine	110.60	5.60	8.27	0.9917	2	Not Reported	10
7-Aminoclonazepam	108.68	4.90	7.56	0.9910	1	†	5
α-PVP	110.6	4.42	5.84	0.9977	0.1	Not Reported	5
Alprazolam	108.03	8.33	5.86	0.9963	0.2	10	2
Amitriptyline	110.39	6.22	7.12	0.9940	0.5	5	5
Amphetamine	88.96	4.79	7.01	0.9989	0.5	Not Reported	5
Benzoylecgonine	89.08	4.2	6.09	0.9985	0.5	5	5
Citalopram	112.77	6.07	9.41	0.9969	0.5	2	2
Clonazepam	108.32	7.39	9.42	0.9941	2	Not Reported	10
Cocaine	97.03	4.8	5.96	0.9989	0.2	2	5
Codeine	108.25	6.04	7.27	0.9981	0.5	50	2
Diazepam	110.83	4.76	7.28	0.9965	0.2	10	2
Ketamine	90.55	2.16	5.76	0.9989	0.2	†	5
LSD	98.3	6.53	6.46	0.9988	0.2	†	5
MDA	86.34	3.73	6.33	0.9968	1	25	5
MDEA	92.58	4.18	8.48	0.9971	0.2	2	5
MDMA	96.22	5.85	6.83	0.9978	0.2	10	5
MDPV	86.86	15.06	5.31	0.9988	0.5	5	5
Mephedrone	104.46	3.62	6.88	0.9974	0.5	†	5
Methamphetamine	92.01	2.25	6.86	0.9985	0.2	10	5
Methylone	99.07	5.98	6.00	0.9962	0.5	5	5
Morphine	104.38	4.38	4.75	0.9977	0.5	Not Reported	2
Nortriptyline	107.8	8.56	6.57	0.9967	1	10	5
Oxycodone	110.53	5.73	5.16	0.9967	0.2	†	5
PCP	98.06	2.67	6.02	0.9981	0.5	10	5
Pseudoephedrine	100.44	6.27	8.74	0.9924	0.2	†	5
Trazodone	104	5.11	9.66	0.9989	0.2	Not Reported	2
Zolpidem	108.98	5.18	8.01	0.9951	0.2	2	5

†-Analytes not evaluated by LDTD-MS/MS

Table A6: Matrix effect and recovery results for 28 drugs and metabolites

Drug Analytes	Matrix 10 ng/mL	Matrix 50 ng/mL	Recovery 10 ng/mL	Recovery 50 ng/mL
6-Acetylmorphine	130	118	13	14
7-Aminoclonazepam	97	89	50	54
α-PVP	100	100	34	38
Alprazolam	102	100	59	65
Amitriptyline	76	78	42	45
Amphetamine	103	102	61	66
Benzoylecgonine	106	107	65	71
Citalopram	91	84	51	57
Clonazepam	103	98	64	62
Cocaine	99	102	62	65
Codeine	104	103	51	59
Diazepam	96	93	56	59
Ketamine	100	100	60	68
LSD	97	96	34	35
MDA	107	109	63	70
MDEA	100	98	62	68
MDMA	105	101	61	66
MDPV	99	101	40	44
Mephedrone	98	102	49	55
Methamphetamine	119	103	63	65
Methylone	99	98	56	63
Morphine	129	121	13	15
Nortriptyline	77	77	41	47
Oxycodone	103	99	51	55
PCP	99	99	48	50
Pseudoephedrine	101	94	58	69
Trazodone	136	91	60	58
Zolpidem	94	88	54	58

Additional Figures

Figure A2: The average response ratios across 6 hematocrit levels, normalized to 45% spotted onto Whatman 903™, Whatman FTA™ DMPK-C and Whatman Elute™ DBS cards

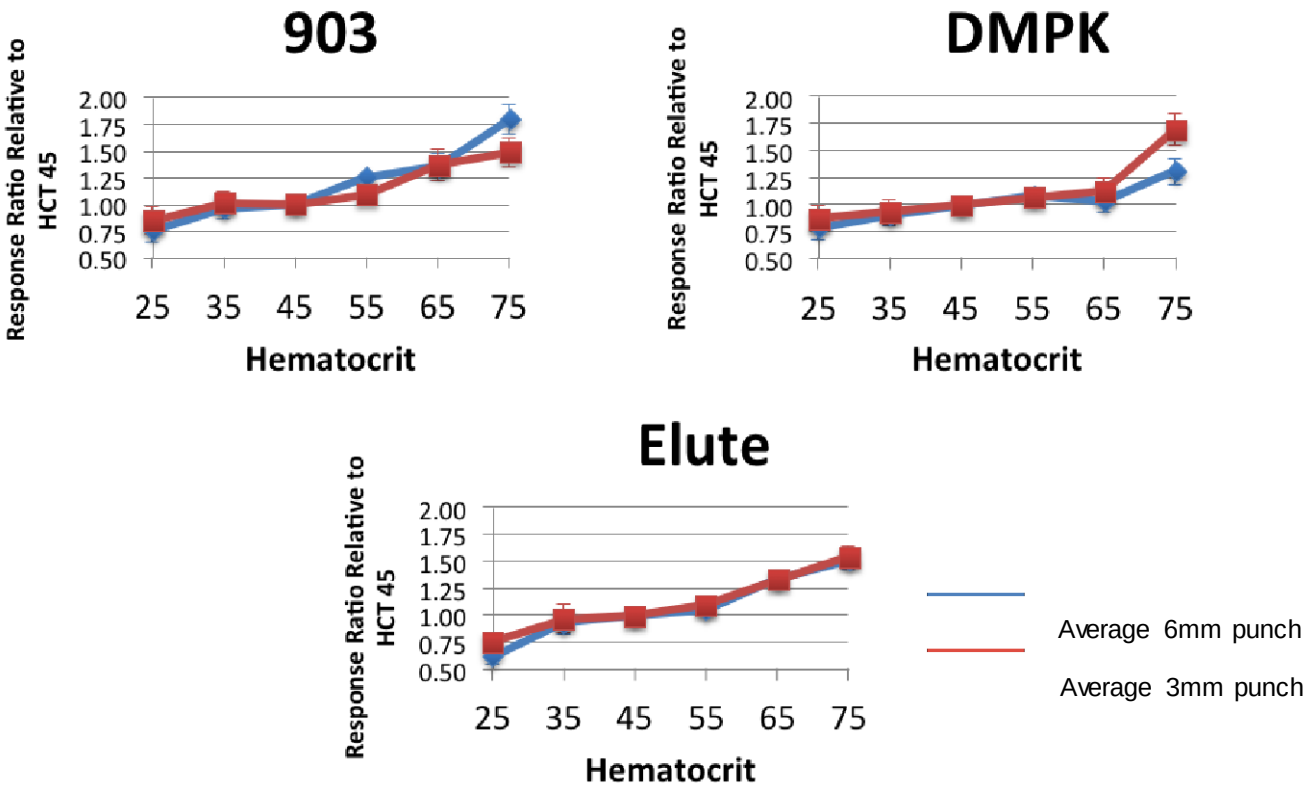

Figure A3: Graph showing the % difference between the concentrations of the samples extracted by DBS and traditional methods

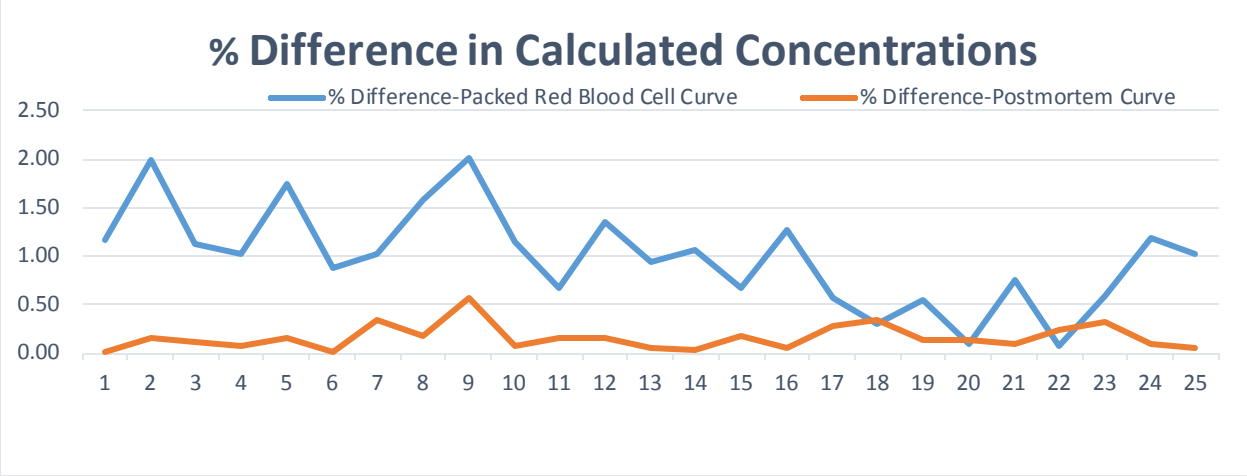

Figure A4: Stability of Group 1 drugs. Y-axis is concentration relative to the concentration on day 0 (baseline) and x-axis is number of days post baseline.

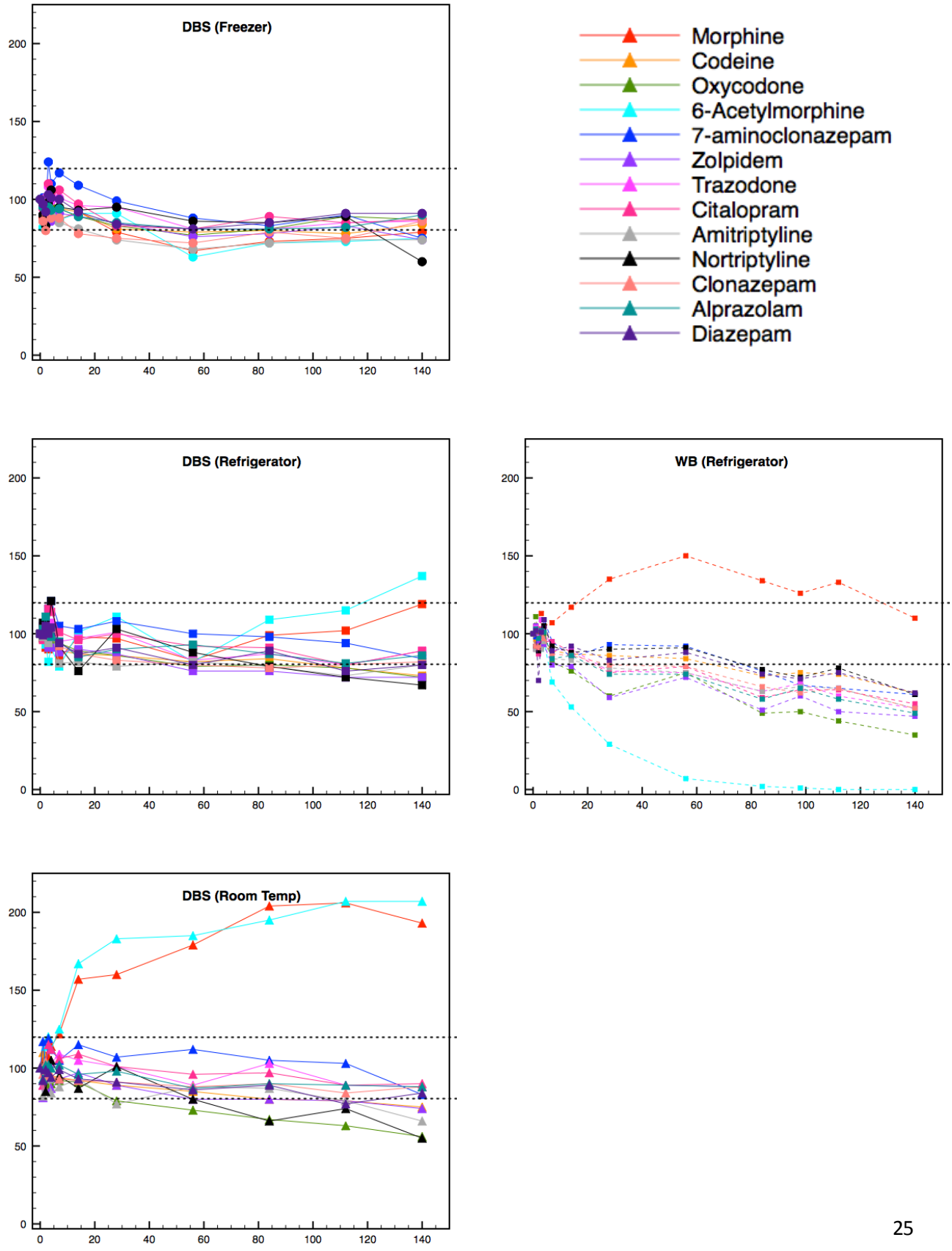

Figure A5: Stability of Group 2 drugs. Y-axis is concentration relative to the concentration on day 0 (baseline) and x-axis is number of days post baseline.