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Automated Desorption, SPE Extraction, and LC/MS/MS Analysis of Dried Blood Spots

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Abstract
The extraction of dried blood spots (DBS) typically involves manual intervention. First, a small disc is punched out of the center of a dried blood spot placed on a DBS card. Following solvent extraction of the sample, it is also common to include further cleanup steps, using solid phase extraction (SPE) to improve detection limits or exchanging solvents for compatibility with subsequent chromatographic separations. Modern analytical labs are looking to automate the process to help reduce solvent usage and to increase sample throughput while ensuring the high quality of the resulting data.

In this report, the complete automation of dried blood spot analysis is demonstrated and the results evaluated. A novel, automated DBS Autosampler (DBS A) automatically inserts DBS cards into a Flow Through Desorption (FTD™) cell in which individual blood spots are rapidly and effectively desorbed. The DBS A is integrated into a complete clean-up and analysis system using online SPE with replaceable cartridges combined with automated injection to an LC/MS/MS system. Automated DBS extraction methods for a variety of analytes from different matrices are examined along with the use of different SPE cartridge sorbents. The resulting precision and accuracy data are provided.

Introduction

Dried blood spot sampling is gaining interest in the scientific community as an alternative technique for acquiring and storing the necessary blood samples for analysis. In addition to the ease of sampling afforded by this technique, DBS also translates into fewer animals used during pre-clinical studies and better quality of data during studies since serial sampling from one animal is possible. The dried samples are easily shipped and enable storage at room temperature, which provides additional cost savings and simplified logistics. Furthermore, samples can be collected in remote locations and shipped to the laboratory without conservation or refrigeration.

After receiving a DBS card in the lab, the time consuming manual sample preparation steps such as spot punching, elution, and centrifugation can be eliminated using the GERSTEL DBSA system shown in Figure 1. Sample desorption from the dried blood spot is performed in a highly reproducible manner and the defined area of the matrix spot is completely desorbed by means of the patented Flow-Through Desorption (FTD™) technology as shown in Figure 2.

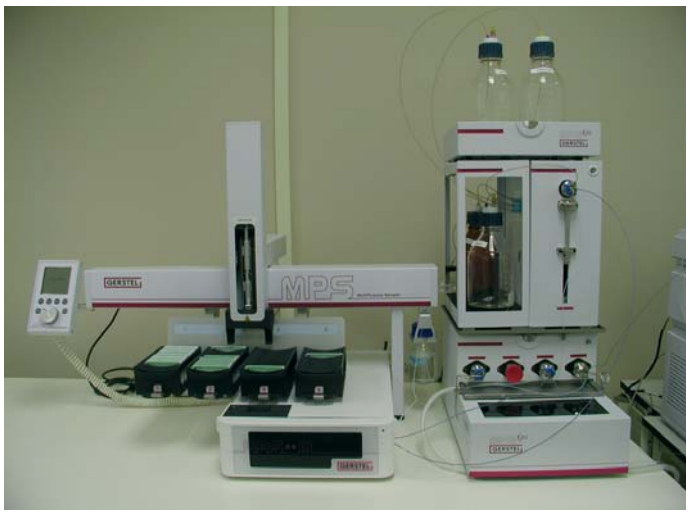

Figure 1. DBSA-SPExos-LC/MS/MS configuration.

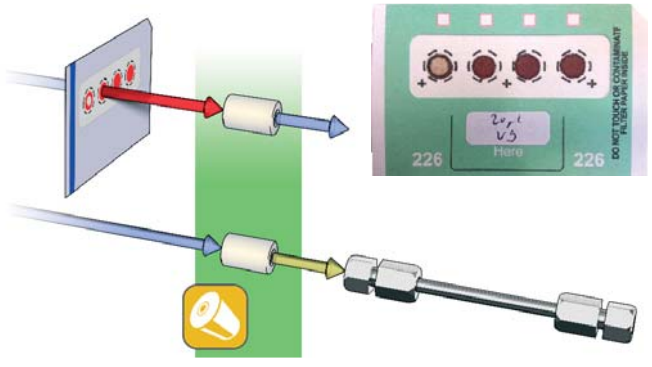

Figure 2. Flow-through desorption technology.

Following desorption, the analytes are automatically transferred onto a user-defined SPE cartridge located inside the GERSTEL SPExos module. The GERSTEL SPExos is an online SPE system with automated cartridge exchange designed for the enrichment and purification of samples before analysis. Using this option, potential matrix interferences from the desorbed blood spots are eliminated prior to analyte determination on the integrated LC/MS/MS system.

As shown in this study, complete automation of DBS-SPE-LC/MS/MS methods is easily set up and controlled using the MAESTRO software.

Experimental

Materials. Ketamine and amitriptyline drug analyte stock solutions were purchased from Cerilliant. Ketoprofen drug analyte stock was purchased from Sigma Aldrich. Intermediate analyte stock solutions were prepared by combining the appropriate analyte stock solutions with methanol resulting in the analyte concentrations needed for method development and evaluation.

Deuterated analogues, d4-ketamine and d3-amitriptyline were purchased from Cerilliant. The deuterated analogue, d3-ketoprofen was purchased from Sigma-Aldrich. A working internal standard stock solution containing all the internal standards used was prepared at a concentration of 10 ng/mL of each, in water.

Rat blood (cat.#031-ABSH-PMG) was purchased from Biochemed. The Intermediate calibration standard and intermediate QC spiking solutions were prepared by making appropriate dilutions of the analyte stock solutions using methanol. Calibration standards in rat blood were then prepared by combining 10 µL of the respective intermediate spiking solution with 990 µL of blank rat blood to give final calibration standard samples at concentrations of 1000, 100, 10, 1, and 0.1 ng/mL respectively. The QC samples were prepared by combining 10 µL of the respective intermediate QC spiking solutions with 990 µL of blank rat blood to give final QC samples at concentrations of 500, 50, and 5 ng/mL.

Bovine blood (cat.#010-ABSH-PMG) was purchased from Biochemed. Intermediate calibration standard and intermediate QC spiking solutions were prepared by making appropriate dilutions of the analyte stock solutions using methanol. Calibration standards in bovine blood were then prepared by combining 10 µL of the respective intermediate spiking solution

with 990 µL of blank bovine blood to give final calibration standard samples having concentrations of 1000, 100, 10, 1, and 0.1 ng/mL. The QC samples were prepared by combining 10 µL of the respective intermediate QC spiking solutions with 990 µL of blank bovine blood to give final QC samples having concentrations of 500, 50, and 5 ng/mL.

All other reagents and solvents used were reagent grade.

Instrumentation. All automated Prep Sequences were performed using the MultiPurpose Sampler (MPS)-based DBSA system shown in Figure 1. The system combines an MPS, GERSTEL DBS, and SPExos Options in combination with an LC/MS/MS system.

Separation of the drug compounds was performed using an Agilent 1290 HPLC with a Poroshell 120, EC-C18, (3.0 x 50 mm, 2.7 µm) column. Analyses of all compounds were performed using an Agilent 6460 Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Samples were directly injected following extraction using the GERSTEL SPExos Option.

Analytical Method LC Method Parameters.

Mobile Phase:	A - 5 mM ammonium formate with 0.05 % formic acid
	B - 0.05 % formic acid in methanol
Gradient:	Initial 5 % B
	0.5 min 5 % B
	1.5 min 30 % B
	3.5 min 60 % B
	5.5 min 70 % B
	6.5 min 95 % B
	7.5 min 95 % B
	7.6 min 5 % B
Pressure:	600 bar
Flowrate:	0.5 mL/min
Run time:	9.0 min
Column Temp.:	55°C

Analytical Method Mass Spectrometer Parameters.

Electrospray + Agilent Jet Stream

Gas Temperature:	350°C
Gas Flow (N_2):	5 L/min
Nebulizer pressure:	35 psi
Sheath Gas Temp:	250°C
Sheath Gas Flow:	11 L/min
Capillary voltage:	4000 V
Nozzle voltage:	500 V

The mass spectrometer acquisition parameters for all compounds are shown in Table 1 along with the qualifier ion transitions. A retention time window value of 2.5 minutes was used for each ion transition being monitored during the course of each of the dynamic MRM experiments.

Table 1. Mass spectrometer acquisition parameters.

Compound	Prec. Ion [m/z]	Prod. Ion [m/z]	Frag. [V]	CE [V]	Cell Acc	Ret. Time [min]	Delta RT [min]	Pol.
Amitriptyline	278.1 278.1	233 117	140	15 15	7 7	4.194	2.5	Pos
D3-Amitriptyline	281.1 281.1	233 117	120	15 20	7 7	4.194	2.5	Pos
D4-Ketamine	242.1 242.1	129 119	102	32 68	7 7	2.500	2.5	Pos
Ketamine	238.1 238.1	220.1 125	105	11 11	7 7	2.500	2.5	Pos
D3-Ketoprofen	256	212	80	2	7	4.495	2.5	Neg
Ketoprofen	253	209	80	2	7	4.495	2.5	Neg

Results and Discussion

The analytes used in this study were chosen because they possess a suitable range of physicochemical properties and are drugs normally found and studied in animals. Ketoprofen (Log $K_{o/w}$=3.0, pK_a=3.88) is a non-steroidal anti-inflammatory (NSAID) drug with veterinary use. Ketamine (Log $K_{o/w}$=3.12, pK_a=7.45) is a dissociative anesthetic and tranquilizer in cats, dogs, horses, and other animals. Amitriptyline (Log $K_{o/w}$=4.95, pK_a=9.76) is a tricyclic antidepressant used to treat separation anxiety, excessive grooming and spraying in certain animals.

After establishing the LC/MS/MS parameters for the analysis of the drug compounds, the first step was to establish the best SPE sorbent for the SPE cleanup step. A method development kit containing SPE cartridges with 8 different SPE sorbents is available, allowing the user to easily screen their respective analytes to determine the best sorbent for the SPE portion of the method. After preparing a solution of the analytes in water, the solution was placed into the internal standard container of the DBSA Option. The software was programmed to condition, equilibrate, and add the analyte solution to each of the respective SPExos cartridges followed by elution of the analytes using the LC/MS/MS gradient. Figure 3 shows the peak responses for Ketoprofen after exposure to either A: A C8 EC-SE SPExos cartridge, B: A C18 HD SPExos cartridge, or C: A Resin GP cartridge. Based on the peak area responses and peak shapes for all three analytes with all SPExos sorbents, the C8 EC-SE SPExos cartridge was selected as the SPExos cartridge to be used for this method.

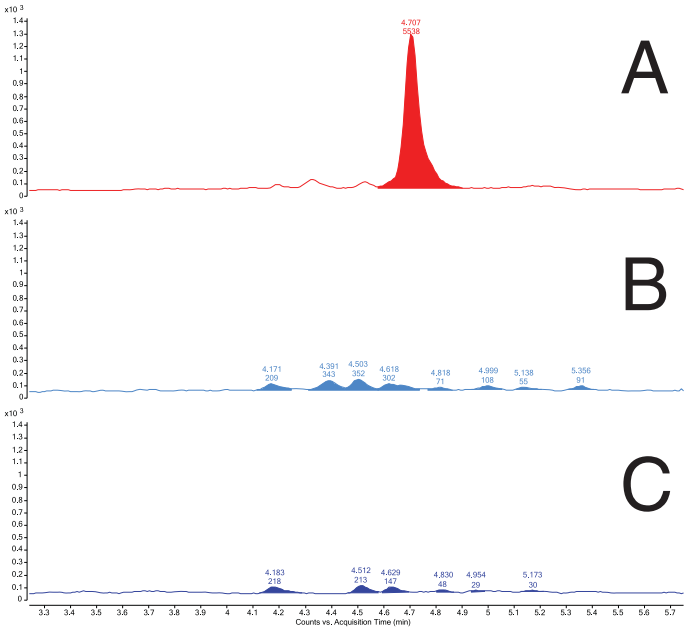

Figure 3. Comparison of ketoprofen results using various SPExos cartridges.

After establishing the proper SPExos cartridge, the next step was to optimize the parameters used to desorb the blood spots from the DBS cards. Since the analytes must be trapped on the SPExos cartridge following desorption, it is important to keep the organic content of the desorption solution as low as possible. Multiple DBS cards (PerkinElmer PKI 226 Bioanalysis Cards) were spotted using 20 µL of rat blood spiked with 10 ng/mL of each analyte. Table 2 contains the details on the desorption parameters used during optimization of the desorption process for the examined analytes as well as the final optimized parameters. During the optimization of the desorption parameters, 1 mL of methanol followed by 1 mL of water were used to clean the DBS system following each desorption process. Throughout the optimization experiments, C8 EC-SE SPExos cartridges were used and were conditioned using 1 mL of methanol followed by 1 mL of water. Each cartridge was washed using 1 mL of water following sample introduction. As shown, the effect of the desorption solution volume used was examined followed by the effect of the desorption solution temperature. Finally, the pH of the desorption solution was optimized for the compounds of interest. Based on the peak area responses and peak shapes for all three analytes, the optimized desorption parameters for this method were found to be 2 mL of a 0.1 % formic acid solution in water at a temperature of 80°C.

Table 2. Optimization of DBS desorption parameters.

Desorption solution	Spot	DHS Desorb	Hot Cap
Volume	1	1 mL H_2O	40°C
	2	2 mL H_2O	40°C
	3	1 mL 10 % MeOH in H_2O	40°C
	4	1 mL H_2O	40°C
Temperature	1	2 mL H_2O	40°C
	2	2 mL H_2O	60°C
	3	2 mL H_2O	80°C
	4	2 mL H_2O	100°C
pH	1	2 mL H_2O	80°C
	2	2 mL 0.1 % formic acid	80°C
	3	2 mL 0.1 % acetic acid	80°C
	4	2 mL 0.1 % NH_4OH	80°C

In order to ensure that sample to sample carry over was minimized, the effect of parameters used to clean the DBSA Option were examined. Carryover was assessed by analyzing dried blood spots from spiked rat blood containing 1000 ng/mL of each analyte followed by a blank DBS card. The analyte peak area determined after elution of a blank DBS card is divided by the analyte peak area found in the spiked dried blood spot to determine carryover for each analyte. The DBS cleaning protocol used following the desorption process was then varied between sets of spiked and blank DBS cards and the carryover results were compared. Table 3 contains the details on the different DBS cleaning parameters used for the carryover experiments as well as the % carryover results from each set. Comparing the results from all carryover tests, a DBS cleaning protocol that used 3 mL of methanol followed by 1 mL of water gave the lowest overall % carryover for all analytes detected.

Table 3. Examination of carryover.

DBS Cleaning Protocol	Ketamine Carryover [%]	Amitriptyline Carryover [%]	Ketoprofen Carryover [%]
1 mL MeOH, 1 mL H_2O	0.508	0.278	5.29
2 mL MeOH, 2 mL H_2O	0.224	0.184	5.73
3 mL MeOH, 3 mL H_2O	0.094	0.114	4.25
3 mL MeOH, 1 mL H_2O	0.067	0.092	5.46
1 mL MeOH, 1 mL 0.1 % FA, 1 mL H_2O	0.093	0.103	25.23
2 mL MeOH, 2 mL 0.1 % FA, 2 mL H_2O	0.210	0.133	16.23
3 mL MeOH, 3 mL 0.1 % FA, 3 mL H_2O	0.082	0.112	16.17
3 mL MeOH, 1 mL 0.1 % FA, 1 mL H_2O	0.334	0.193	11.35

The lower limit of quantitation of ketamine was determined to be 0.1 ng/mL in both rat and bovine blood. The lower limits of quantitation for amitriptyline and ketoprofen were determined to be 10 ng/mL in both rat and bovine blood. Regression analysis for all analytes resulted in R^2 values of 0.99 or greater. A representative calibration curve for ketamine in dried rat blood spots is shown in Figure 4 and in dried bovine blood spots is shown in Figure 5.

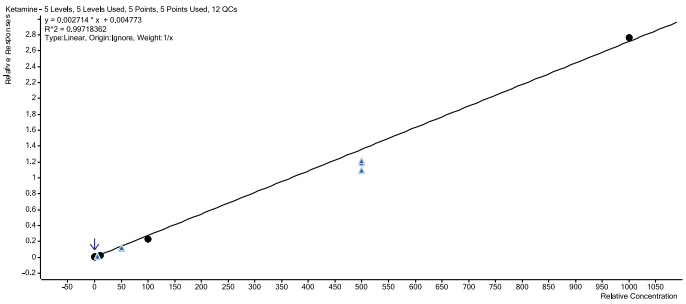

Figure 4. Representative calibration curve results for ketamine following automated DBS-SPExos-LC/MS/MS analysis of dried rat blood spots.

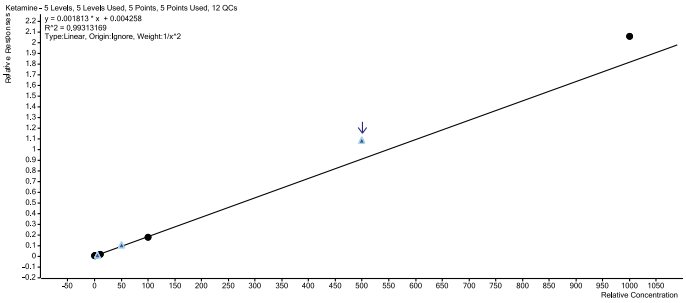

Figure 5. Representative calibration curve results for ketamine following automated DBS-SPExos-LC/MS/MS analyses of dried bovine blood spots.

Following the automated DBS-SPExos-LC/MS/MS analysis of dried rat blood samples, the accuracy and precision measured for ketamine, amitriptyline, and ketoprofen were measured by extracting replicate (n=4) QC samples at various concentrations. The number of QC samples evaluated depended on the resulting limit of quantitation of the analyte. Table 4 shows the resulting accuracy and precision data for all compounds extracted from dried rat blood samples. Accuracy data averaged 86.8 % for ketamine, 87.2 % for amitriptyline, and 104 % for ketoprofen. Precision data (% CV) averaged 6.19 % for ketamine, 4.62 % for amitriptyline, and 6.24 % for ketoprofen.

Table 4. Results of automated DBSA-SPExos-LC/MS/MS analysis of ketamine from dried rat blood spots.

QC Sample	Ketamine		Amitriptyline		Ketoprofen	
	Concentration found [ng/mL]	Rec. [%]	Concentration found [ng/mL]	Rec. [%]	Concentration found [ng/mL]	Rec. [%]
QC 5 ng/mL 1	5.55	111				
QC 5 ng/mL 2	4.71	94.1				
QC 5 ng/mL 3	4.26	85.1				
QC 5 ng/mL 4	4.21	84.3				
mean	4.68	93.6				
SD	0.539					
% CV	11.5					
QC 50 ng/mL 1	41.2	82.3	43.0	85.9	50.7	101
QC 50 ng/mL 2	43.0	85.9	45.6	91.3	52.3	105
QC 50 ng/mL 3	40.3	80.6	40.7	81.3	49.5	99.1
QC 50 ng/mL 4	40.7	81.4	43.1	86.3	54.5	109
mean	41.3	82.6	43.1	86.2	51.8	104
SD	1.02		1.76		1.87	
% CV	2.47		4.08		3.61	
QC 500 ng/mL 1	403	80.7	417	83.5	435	87.0
QC 500 ng/mL 2	445	88.9	468	93.6	535	107
QC 500 ng/mL 3	401	80.3	420	83.9	543	109
QC 500 ng/mL 4	437	87.4	458	91.7	541	108
mean	422	84.3	441	88.2	514	103
SD	19.4		22.7		45.5	
% CV	4.61		5.15		8.86	

Following the automated DBS-SPExos-LC/MS/MS analysis of dried bovine blood samples, the accuracy and precision measured for ketamine, amitriptyline, and ketoprofen were measured by extracting replicate (n=4) QC samples at various concentrations. The number of QC samples evaluated depended on the resulting limit of quantitation of the analyte. Table 5 shows the resulting accuracy and precision data for all compounds from dried bovine blood samples. Accuracy data averaged 112 % for ketamine, 109 % for amitriptyline, and 102 % for ketoprofen Precision data (% CV) averaged 4.45 % for ketamine, 6.16 % for amitriptyline, and 5.74 % for ketoprofen.

Table 5. Results of automated DBSA-SPExos-LC/MS/MS analysis of ketamine from dried bovine blood spots.

QC Sample	Ketamine		Amitriptyline		Ketoprofen	
	Concentration found [ng/mL]	Rec. [%]	Concentration found [ng/mL]	Rec. [%]	Concentration found [ng/mL]	Rec. [%]
QC 5 ng/mL 1	5.81	116				
QC 5 ng/mL 2	4.59	91.7				
QC 5 ng/mL 3	5.37	107				
QC 5 ng/mL 4	4.61	92.2				
mean	5.09	102				
SD	0.521					
% CV	10.2					
QC 50 ng/mL 1	57.5	115	55.7	111	51.6	103
QC 50 ng/mL 2	57.2	114	52.0	104	45.4	90.8
QC 50 ng/mL 3	58.9	118	55.5	111	51.3	103
QC 50 ng/mL 4	54.4	109	49.2	98.4	48.9	97.8
mean	57.0	114	53.1	106	49.3	98.6
SD	1.63		2.68		2.47	
% CV	2.86		5.05		5.00	
QC 500 ng/mL 1	596	119	499	100	546	109
QC 500 ng/mL 2	597	119	546	106	492	98.5
QC 500 ng/mL 3	600	120	597	119	497	99.3
QC 500 ng/mL 4	596	119	596	119	574	115
mean	597	119	559	112	527	105
SD	1.72		40.7		34.2	
% CV	0.288		7.27		6.49	

Conclusions

As a result of this study, we were able to show:

- Automated dried blood spot extraction methods were developed very efficiently using a combined GERSTEL DBSA and SPExos system under MAESTRO software control.
- Drug compounds of veterinary interest were successfully extracted from dried blood spot samples using an automated procedure on a DBS-SPExos system coupled with an Agilent 6460 Triple Quadrapole Mass Spectrometer used for LC-MS/MS determination.
- Linear calibration curves were established using both dried rat blood and dried bovine blood resulting in R^2 values 0.99 or greater.
- The DBS-SPExos-LC/MS/MS method proved to be accurate and precise. For dried rat blood spots, accuracy data averaged 91.7 % (range: 82.6 % - 104 %) and precision data averaged 5.75 %CV (range: 2.47 % -11.5 %) for all compounds analyzed. For dried bovine blood spots, accuracy data averaged 108 % (range: 98.6 % - 119 %) and precision data averaged 5.31 %CV (range: 0.288 % -10.2 %) for all compounds analyzed.

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