GX-274 ASPEC®: Automated Extraction of Glucocorticoid and Nonsteroidal Anti-Inflammatory Drugs from Horse Urine

APPLICATION NOTE AN1004

APPLICATION BENEFITS

Glucocorticoid and nonsteroidal anti-inflammatory compounds were isolated from the same sample by automating multiple elution steps. TRILUTION® LH software allowed easy automation of manual SPE methods that fit into existing workflows and provide reproducible and traceable results. A four-probe GX-274 ASPEC® instrument provides sufficient throughput to prepare samples for two HPLC/MS/MS systems in parallel, reducing the number of instruments required in the lab.

SOLUTIONS

Accurate and rapid detection of prohibited substances present in complex biological matrices is crucial for testing laboratories. Robust instrumentation, a flexible software interface that meets the needs of the end user, reproducible methods and traceability are all needed in regulated environments.

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ABSTRACT

Three glucocorticoids and three nonsteroidal anti-inflammatory drugs were detected simultaneously from horse urine using the Gilson GX-274 ASPEC® system with two step extractions and LC/MS/MS selected reaction monitoring (SRM).

INTRODUCTION

Equine drug testing laboratories face the challenge of rapidly and accurately detecting drugs and other foreign substances that are prohibited in horse racing.[1] Automation with the Gilson GX-274 ASPEC® system provides a reproducible,

Figure 1
Gilson GX-274 ASPEC® system

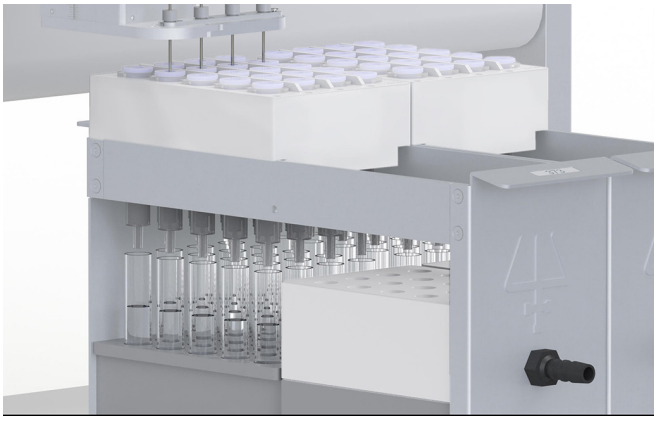

Figure 2
The system is equipped with four probes, permitting four samples to be processed simultaneously with automated solid phase extraction.

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documentable method for sample preparation and fits into workflows for compound detection and quantitation (Figures 1 and 2). With four probes, the GX-274 ASPEC system provides higher throughput while maintaining reliability and providing a flexible software interface for development of methods that meet the needs of the research or testing lab.

MATERIALS AND METHODS

Solid Phase Extraction

A Gilson GX-274 ASPEC® system was used for the extraction.[2] The solid phase extraction (SPE) cartridges used in this study were Presep® RPP-SAX (60 µm particle, 60 mg/3 mL) obtained from Wako Pure Chemicals. The Presep RPP-SAX cartridges were pre-conditioned with 2 mL of methanol followed by 2 mL of 50 mmol/L ammonium formate.

The sample processing workflow is shown in Figure 3. Internal standards made up in 1mL of 1 mol/L acetate buffer (pH 5.0) and 100 µL of beta glucuronidase from *Pomacea canaliculata* solution were added to 1.0 mL of horse urine followed by incubation for 30 minutes at 60°C. After cooling the mixture to ambient temperature, 4 mL of 0.3 mol/L sodium dihydrogen phosphate containing 0.01 mol/L EDTA2Na was added. The mixture was filtered with glass fiber filter paper and a 4.5 mL aliquot was added to a pre-conditioned Presep RPP-SAX SPE column. The SPE column was washed with 3 mL of water/methanol (95:5) and eluted with 6 mL of t-butyl methyl ether. The resulting eluate was termed Eluate G and contained the glucocorticoid.

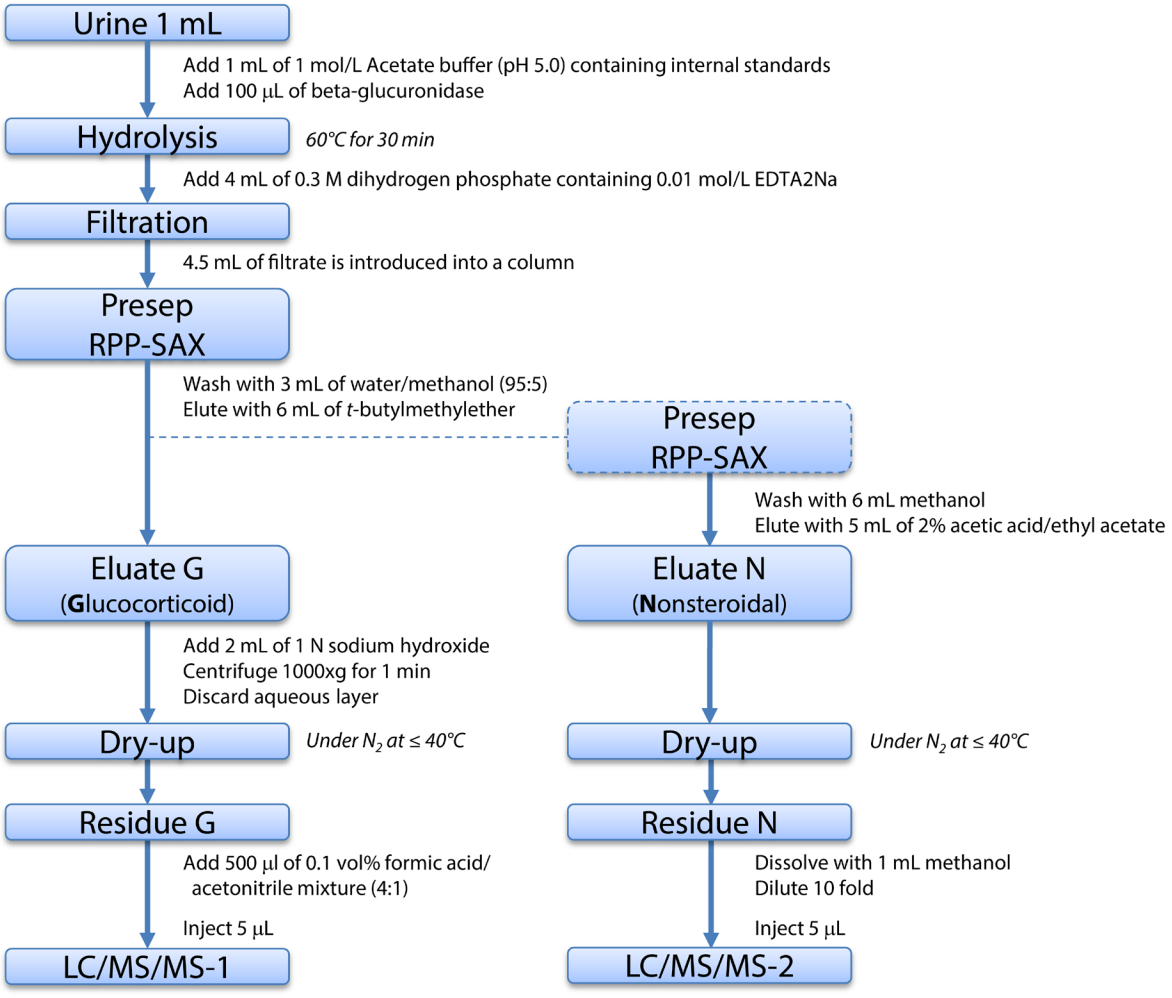

Figure 3
Schematic of the procedure used for two step sample preparation for glucocorticoid and nonsteroidal anti-inflammatory drug detection.

After the elution, the column was washed with 6 mL of methanol and eluted with 5 mL of 2% (volume) acetic acid/ethyl acetate. The resulting eluate was termed Eluate N and contained the nonsteroidal anti-inflammatory drug fraction.

Nonsteroidal anti-inflammatory (indomethacin, diclofenac, flunixin) and glucocorticoid (triamcinolone acetonide, betamethasone, methylprednisolone) reference standards were spiked into blank urine as samples and extracted with the method described.

MS Analysis

To the Eluate G, 2 mL of 1 mol/L sodium hydroxide was added and mixed for 1 min. The solution was then centrifuged for 1 min at 1000xg. The aqueous layer was removed and the t-butyl methyl ether layer was evaporated using a nitrogen stream ≤ 40°C. The residual precipitate, called Residue G, was dissolved with 500 µL of 0.1 vol% formic acid/acetonitrile (4:1) mixture. This solution was used as the sample for LC/MS/MS-1.

Eluate N was dried under the nitrogen stream at ≤ 40°C. The residual precipitate was called Residue N. The Residue N was dissolved with 1000 µL of methanol. From this, 100 µL of aliquot was taken. To the aliquot, 900 µL of methanol was added. The mixture was used as the injection sample for LC/MS/MS-2 analysis (Table 2).

The injection sample for LC/MS/MS-1 contained dexamethasone-d4, hydrocortisone-d3 and the injection sample for LC/MS/MS-2 contained N-phenylanthranilic acid at 0.2 ng/mL, 10 ng/mL, and 10 ng/mL, respectively, as internal standards.

A Prominence HPLC (Shimadzu) in combination with a 4000 QTRAP® MS/MS (AB SCIEX) was used for the LC/MS/MS analysis.

Table 1:
LC/MS/MS conditions for glucocorticoid analysis of Residue G

HPLC: PROMINENCE LC-20 (SHIMADZU)									
Column	Atlantis T3(Waters), L: 50 mm, ID: 2.1mm, particle size: 3µm								
Column temperature	40°C								
Mobile phase	A = 0.1vol% formic acid, B=acetonitrile								
Gradient profile	Time (min)	0	0.5	6.0	6.01	7.5	7.51	7.6	9.5
	A(%)	80	80	50	2	2	80	80	80
	B(%)	20	20	50	98	98	20	20	20
	Flowrate (mL/min)	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.4
Ionization accelerator	Acetonitrile with 10mM glycerol was added to column eluate at 0.1mL/min.								
Injection volume	5 µL								
MASS SPECTROMETER: 4000 Q TRAP (AB SCIEX)									
Ionization method:	Electrospray ionization								
Ion polarity:	Negative polarity mode								
Ionization temperature:	600°C								
Ionization spray voltage:	-4500V								

Table 2:
LC/MS/MS conditions for nonsteroidal anti-inflammatory drug analysis of Residue N

HPLC: PROMINENCE LC-20 (SHIMADZU)								
Column	Atlantis T3(Waters), L: 50 mm, ID: 2.1mm, particle size: 3µm							
Column temperature	40°C							
Mobile phase	A = 0.1vol% formic acid, B=acetonitrile							
Gradient profile	Time (min)	0	0.5	5.5	7.5	7.51	7.6	9.0
	A(%)	80	80	2	2	80	80	80
	B(%)	20	20	98	98	20	20	20
	Flowrate (mL/min)	0.2	0.2	0.2	0.2	0.2	0.4	0.4
Ionization accelerator	Acetonitrile with 10mM glycerol was added to column eluate at 0.1mL/min							
Injection volume	5 µL							
MASS SPECTROMETER: 4000 Q TRAP (AB SCIEX)								
Ionization method:	Electrospray ionization							
Ion polarity:	Negative polarity mode							
Ionization temperature:	600°C							
Ionization spray voltage:	-4500V							

RESULTS AND DISCUSSION

Examples of the drug detection using this system are shown in Figure 4. For all drugs detected, spiked urine gave drug peaks' relative retention times within ± 2% of those of the peaks of reference standards-only injections. There were no contaminant peaks which hindered the detection of the desired peaks from the analytes.

The detection sensitivity was evaluated by testing the recovery of reference standards (n=5) (Table 3). Glucocorticoids demonstrated a 67%–73% recovery, while the nonsteroidal anti-inflammatory drugs were recovered at 81%–97%.

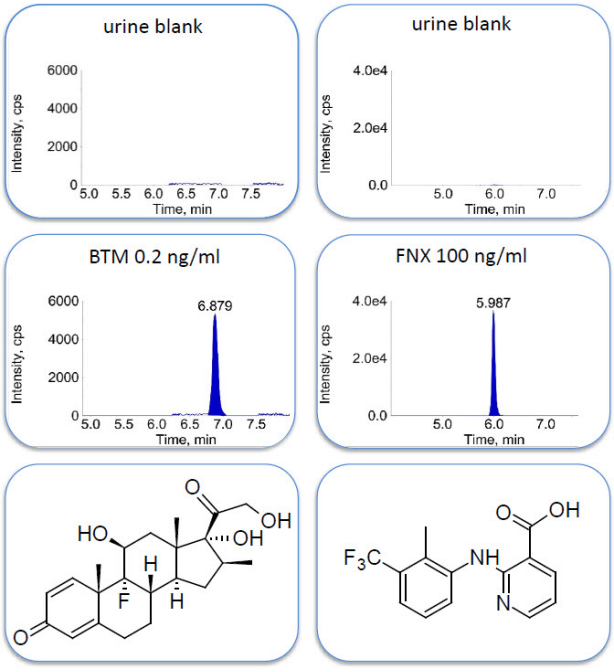

Figure 4
Examples of glucocorticoid Betamethasone (BTM) detection (left) and nonsteroidal anti-inflammatory drug Flunixin (FNX) detection (right).

Table 3:
Detected drugs and selected internal standard ions with recovery rates

NONSTEROIDAL ANTI-INFLAMMATORY DRUGS	PRECURSOR ION (m/z)	SELECTED ION (m/z)	SPIKED CONC. (ng/mL)	%RECOVERY	%RSD (N=5)
Detected Drugs					
Indomethacin	356.0	312.0	100	81	2.2
Diclofenac	294.0	214.0	50	97	4.8
Flunixin	295.0	234.0	100	96	3.4
Internal Standard					
N- phenylanthranilic acid	212.0	168.0			
GLUCOCORTICOIDS	**PRECURSOR ION (m/z)**	**SELECTED ION (m/z)**	**SPIKED CONC. (ng/mL)**	**%RECOVERY**	**%RSD (N=5)**
Detected Drugs					
Triamcinolone acetonide	479.3	337.1	5	73	5.9
Betamethasone	437.2	361.0	0.2	68	3.3
Methylprednisolone	419.2	343.1	10	67	3.0
Internal Standard					
Dexamethasone-d4	441.1	362.9			
Hydrocortisone-d3	410.2	127.9			

CONCLUSIONS AND BENEFITS

Automating multiple elution steps into different collection tubes allows parallel extractions of glucocorticoid and nonsteroidal anti-inflammatory compounds from the same sample tube.

With four probes, just one GX-274 ASPEC® instrument provides sufficient throughput to prepare samples for two HPLC/MS/MS systems in parallel. This reduces the number of instruments required in the lab, saving money and bench space.

The flexible TRILUTION® LH software allows easy translation of manual SPE methods to fit into your personalized workflows for compound detection and quantitation.

The washable probes offer an economical alternative to disposable tips and are proven to avoid cross contamination.

Automated SPE provides verifiable results that can be tracked and trusted.

REFERENCES

1. Association of Racing Commissioners International, Inc. Uniform Classification Guidelines for Foreign Substances. Version 9.00 revised April 2015. http://arcicom.businesscatalyst.com/
2. Gilson Guide to SPE Automation. 2015. http://www.gilson.com/Resources/Gilson_Guide%20to_SPE_Automation_2015.pdf
3. Wako Pure Chemicals Industry, Ltd. Page accessed 11/6/15. http://www.wako-chem.co.jp/english/labchem/product/analytical/presep_rpp_sax/index.htm

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