APPLICATIONS

Extraction of THC-COOH and Barbiturates from Urine using Novum™ Simplified Liquid Extraction

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Introduction

In this technical note we develop a Simplified Liquid Extraction (SLE) application for barbiturates and THC-COOH from a urine matrix containing β-glucuronidase followed by LC-MS/MS method using Kinetex® EVO C18 LC column and SCIEX API 4000™ in negative mode electrospray ionization (ESI-).

Materials

Secobarbital, Amobarbital, Phenobarbital, Butabital, Pentobarbital, Pentobarbital-D5. 11-nor-9-Carboxy-Δ9-THC (COOH-THC) and 11-nor-9-Carboxy-Δ9-THC-D3 (COOH-THC-D3) standards were purchased from Cerilliant® (Round Rock, TX). Campbell Beta Glucuronidase Enzyme was purchased through Campbell Science Product, 100,000 units/mL (Rockford, IL). Formic acid was purchased from Sigma-Aldrich® (St. Louis, MO). HPLC-grade acetonitrile, methanol, methyl tert-butyl ether, ethyl acetate and hexane were purchased from Honeywell™ (Morris Plains, NJ).

Experimental Conditions

For all elution solvents, the following sample pretreatment, SLE, and LC conditions were followed, with the elution solvent being the only variant.

Sample Pretreatment

Each sample was comprised of 150 µL of urine, 25 µL β-glucuronidase with 73 µL of 0.1M Ammonium Acetate buffer, pH 4 and 2 µL of barbiturate standards with THC-COOH. This 250 µL was then combined with 200 µL of 1 % formic acid in water solution.

SLE Protocol

96-Well Plate:	Novum SLE MAX
Part No.:	8E-S138-5GA
Load:	Pretreated sample and pulse vacuum (~5" Hg) for 5-10 seconds or until sample has complete entered the sorbent
Elute:	2 x 900 µL aliquots of 7 test solvents (see list below) followed by a short pulse 5" Hg to initiate flow. Allow the remaining solvent to flow via gravity. At the completion of the second aliquot, apply vacuum at 5" Hg for 15 seconds to complete the extraction
Dry down:	Evaporate eluate to dryness @ room temperature under a gentle stream of nitrogen
Reconstitute:	In 200 µL of methanol/water (50:50) containing 1 % NH_4OH and 100 ng/mL of COOH-THC-D3 and 250 ng/mL of Pentobarbital-D5100 ng/mL

Tested Elution Solvents

1. MTBE (Methyl Tert-Butyl Ether)
2. Ethyl Acetate
3. Hexane/Ethyl acetate (1:1)
4. Hexane/Ethyl acetate (1:3)
5. Hexane/MTBE (1:1)
6. Hexane/MTBE (1:3)
7. Hexane/Ethyl acetate (3:1)

LC Conditions

Analytical Column: Kinetex 2.6 µm EVO C18 100Å
Dimension: 50 x 2.1 mm
Part No.: 00B-4725-AN
Recommended SecurityGuard™ ULTRA Cartridge: AJ0-7844
Mobile Phase: A: 10 mM Ammonium bicarbonate, pH 9
B: Acetonitrile

Gradient:

Time (min)	B (%)
0	5
2	15
5	20
5.01	60
6	60
6.1	5
7.5	5

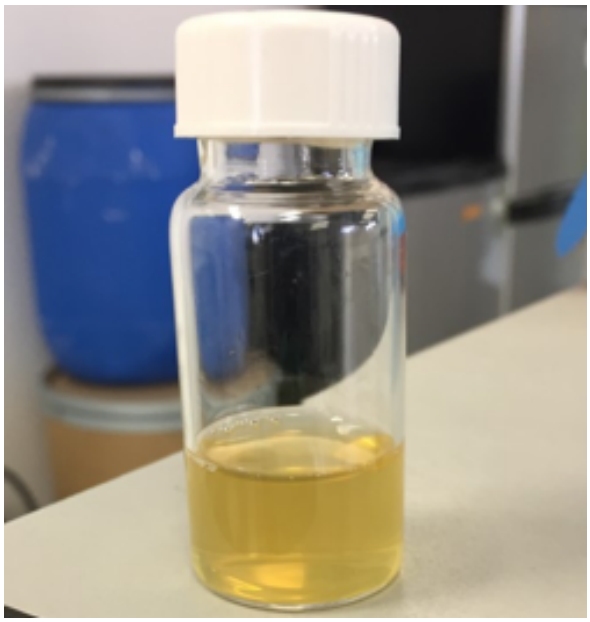
Flow Rate: 0.5 mL/min
Temperature: Room temperature
Injection Volume: 2 µL
Instrument: Agilent® 1200 LC System
Detection: MS/MS API 4000 (SCIEX), ESI+

Revision: 0
PHEN-RUO-00032

Results and Discussion

While all seven solvents were screened for the SLE protocol, recovery data was only generated for the two samples that were visually the cleanest after dry down and reconstitution. **Figure 1** shows the sample vat before it is extracted using Novum™ SLE. **Figure 2** shows the reconstituted samples using the following extracting solvents: MTBE (Vial A), EtOAc (Vial B), Hexane/MTBE (1:3) (Vial C) and the Hexane/EtOAc (Vial D). Vials C and D appear clearer, therefore presumptively cleaner than Vials A and B. This effect becomes more apparent with dirtier and more concentrated urine samples.

Figure 1. Sample prior to extraction



Hexane/MTBE (1:3)

Historically, MTBE provides acceptable recoveries for these classes of compounds, but often times provides mediocre clean up. By cutting the MTBE with a 25 % volume of hexane, we are able to drastically improve the cleanup of the sample while maintaining the recovery values that are shown in **Table 1**. This extracting solvent is the best choice to achieve the highest recovery.

Hexane/EtOAc (3:1)

It has been discussed in previous technical notes that using straight hexane, or any non-oxygen containing solvent by itself with Novvum SLE plates does not usually produce acceptable results.The addition of an oxygen containing solvent is usually required to help solvate the sorbent in order to promote an efficient extraction. Moreover, hexane is often so non-polar that many analytes are not soluble in it, which can be good for maintaining sample cleanliness, but not for recovery. Therefore, the addition of ethyl acetate is used to reduce the solvent's hydrophobicity to allow polar compounds (i.e. barbiturates) to be extracted while also functioning as an oxygen containing modifier to further improve extraction efficiency.

Additionally, this solvent mixture dries down faster than ethyl acetate, while providing the acceptable recovery values (**Table 1**). This solvent is the best choice for those who are interested in good recovery with fast dry down. **Figure 3** shows a chromatogram for this extract using this solvent.

Figure 2. Extracted samples post dry down and reconstitution in 200 µL of solvent for visual comparison. Solvents shown are: A) MTBE, B) EtOAc, C) Hexane/MTBE (1:3), D)Hexane/EtOAC (3:1).

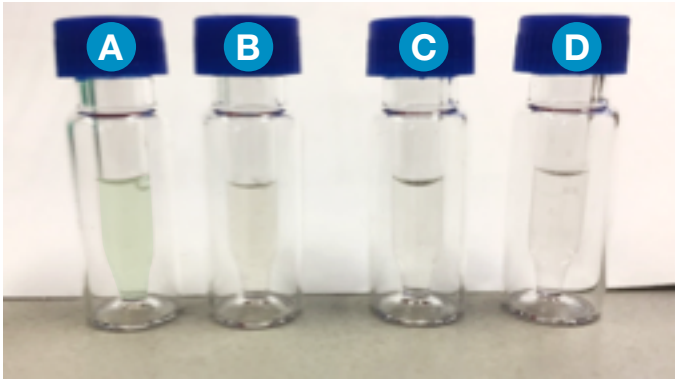


Table 1. Recovery values of Barbiturates and THC-COOH for the two visual clean solvents: Hexane/MTBE (1:3) and Hexane/EtOAc (3:1).

	Hexane/ MTBE (1:3)		Hexane/ EtOAc (3:1)	
	Average Recovery	%CV (N=4)	Average Recovery	%CV (N=4)
Amobarbital	105%	8	93%	4
Pentobarbital	95%	9	87%	4
Butabital	98%	11	93%	7
Phenobarbital	95%	6	85%	8
Secobarbital	103%	12	88%	13
THC-COOH	89%	6	81%	12

Basic mobile phase was selected in order to provide a stronger source of ionization in negative mode. We selected Kinetex EVO C18 for stable separation at pH 9. While the THC-COOH represented here was spiked at 100 ng – and 10 ng spike of THC-COOH was also performed; unfortunately due to system limitations, the peaks for this were merely qualitative, and since they did not meet the required threshold for quantitation (10:1 signal-to-noise ratio), integration was not performed and recovery was not calculated. However, relative approximations between pre and post spike responses, coupled with the results presented herein, indicate that the method should scale to a lower concentration of THC-COOH. An example of the neat standards are show in **Figure 4,** which illustrates the required separation of the pentobarbital and amobarbital isomers**.**

Figure 3. Representative chromatogram for analytes extracted using Hexane/EtOAc (3:1). Peaks in order of elution: Phenobarbital (1.15 min), Butabital (2.04 min), Pentobarbital (3.21 min), Amobarbital (3.48 min), Secobarbital (4.12 min), THC-COOH (6.05 min)

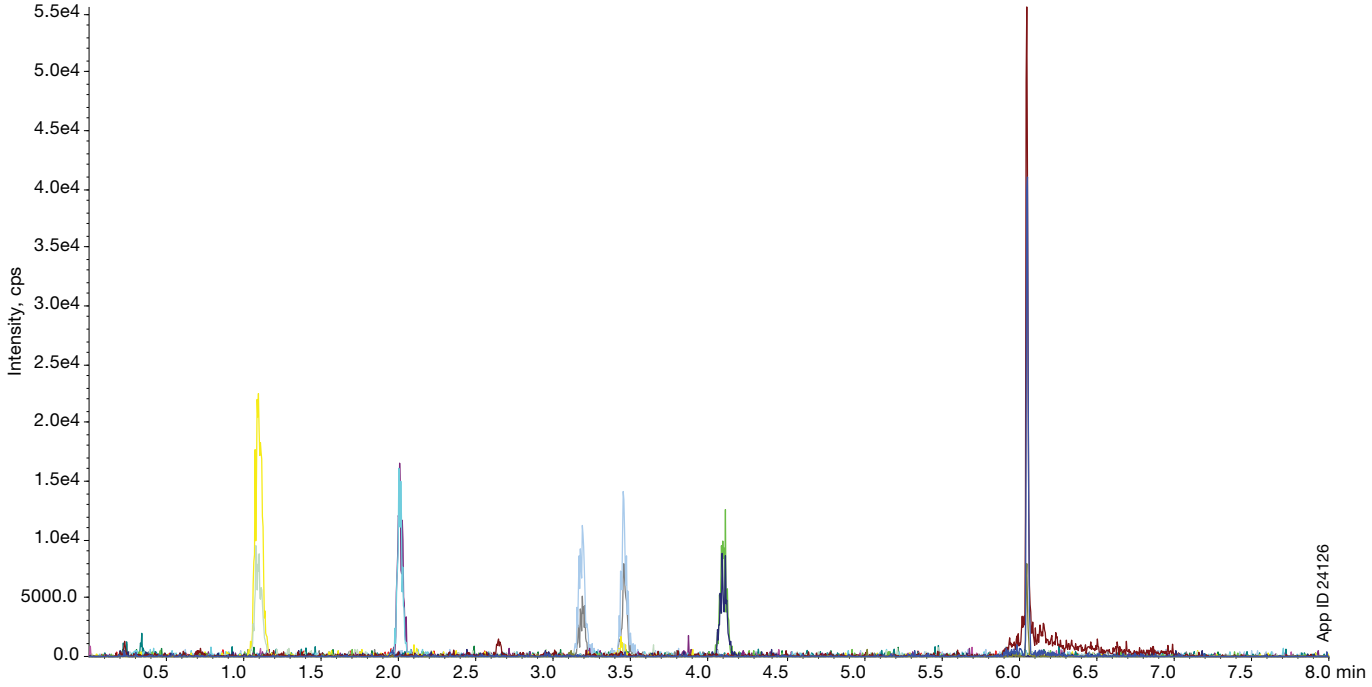
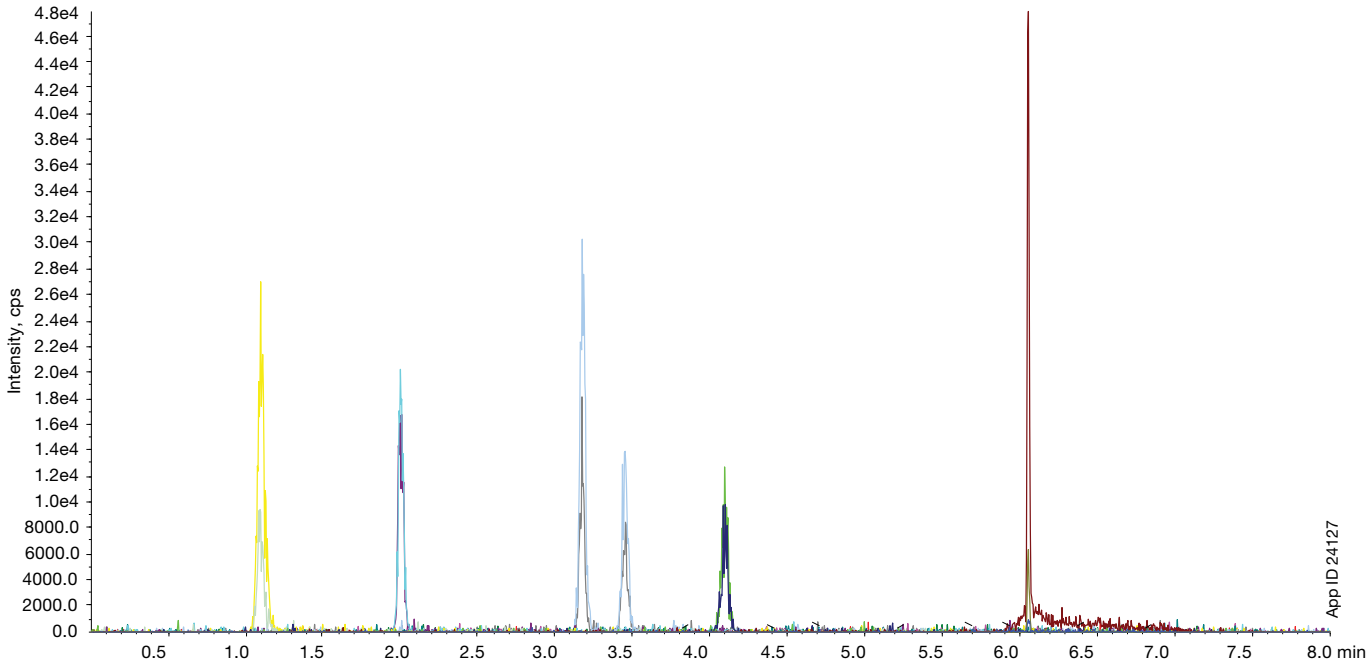


Figure 4. Representative chromatogram of neat standards of barbiturates and THC-COOH. Peaks in order of elution: Phenobarbital (1.15 min), Butabital (2.04 min), Pentobarbital (3.21 min), Amobarbital (3.48 min), Secobarbital (4.12 min), THC-COOH (6.05 min)



APPLICATIONS

Conclusion

In this technical note we identified the best elution solvents for the simultaneous extraction of barbiturates and THC-COOH from urine. Using Novum SLE, coupled with Hexane/MTBE extracting solvent provided a visually clean extract and good recovery for all analytes. Hexane/EtOAc provides slightly less recovery with the same visual cleanliness, but with the added benefit of fast dry down.

Ordering Information

Kinetex® LC Columns

2.6 µm Minibore Columns (mm)					SecurityGuard™ ULTRA Cartridges
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
EVO C18	00A-4725-AN	00B-4725-AN	00D-4725-AN	00F-4725-AN	AJ0-9298

Novum™ Simplified Liquid Extraction (SLE)

96-Well Plates

Part No.	Description	Unit
8E-S138-FGA	Novum SLE MINI 96-Well Plate	1/Box
8E-S138-5GA	Novum SLE MAX 96-Well Plate	1/Box

Tubes

Part No.	Description	Unit
8B-S138-FAK	Novum SLE 1 cc tubes	100/box
8B-S138-5BJ	Novum SLE 3 cc tubes	50/box
8B-S138-JCH	Novum SLE 6 cc tubes	30/box
8B-S138-KDG	Novum SLE 12 cc tubes	20/box

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