Application Note

Clinical Research

A Fast Analytical LC/MS/MS Method for the Simultaneous Analysis of Barbiturates and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-A) in Urine

Using ESI negative ionization mode and alternating column regeneration

Authors

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Abstract

Most of the compounds in large drug panels are analyzed using positive ionization mode. However, barbiturates and 11-nor-9-carboxy-Δ^9-THC (THC-A) perform better using electrospray ionization (ESI) in negative mode with the mobile phase pH favorable for negative ionization. This work developed a fast analytical method combining eight barbiturates and THC-A in a single analysis using alternating column regeneration (ACR) to increase sample throughput. Moving the analytes preferring negative ionization into a separate test increases the analytical sensitivity of the compounds, allowing for greater research capabilities. The simple sample preparation techniques used provided rapid analysis, good analytical sensitivity, and quantitation over a wide dynamic range.

This analysis used an Agilent 6470 triple quadrupole mass spectrometer with Agilent Jet Stream technology in ESI negative ionization mode and an Agilent Infinity II 1290 UHPLC system. A second pump and 2-position 10-port switching valve were added to facilitate use of the ACR. A 100 µL aliquot of human urine was used for the analysis of barbiturates and THC-A. Chromatographic separation of the analytes was achieved in less than 3 minutes using a gradient method composed of a H_2O:acetonitrile mixture with 5 mM ammonium acetate and two Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 1.9 µm columns. Quantitative analysis was performed using multiple reaction monitoring (MRM) transition pairs for each analyte and an internal standard in the negative ionization mode. The isobaric pair, amobarbital and pentobarbital, were not separated under these chromatographic conditions. Good linearity and reproducibility were obtained for the concentration range from 5 to 1,000 ng/mL with a coefficient of determination >0.995 for all analytes. Excellent reproducibility was observed for all analytes (CV <15 %). A fast, specific, and accurate quantitative LC/MS/MS analytical method was developed and verified for the simultaneous measurement of barbiturates and THC-A in urine.

Introduction

Compounds in large drug panels are analyzed using positive ionization mode. However, barbiturates and 11-nor-9-carboxy-Δ^9-THC (THC-A) perform better in negative ionization mode using a separate assay with the mobile phase pH favorable for negative ionization. Included in the analysis were eight barbiturates and THC-A (Figure 1). barbiturates included: amobarbital, butabarbital, butalbital, methohexital, pentobarbital, phenobarbital, hexobarbital, and secobarbital. This work developed a fast analytical method combining barbiturates and THC-A in a single analysis using alternating column regeneration (ACR) to increase sample throughput. This analytical method used the ability of LC/MS/MS to detect compounds over a wide range of concentrations simultaneously. The calibration concentrations ranged from 0.1 ng/mL to 5,000 ng/mL. The standard curve preparation was generated using matrix-matched standards, diluting 1:10 and injecting into the LC/MS/MS system. The methodology was developed on an Agilent 1290 Infinity II UHPLC and an Agilent 6470 LC/TQ mass spectrometer with a 5-minute analytical gradient. ACR reduced the analysis time by 26 %, to 3.7 minutes injection to injection.

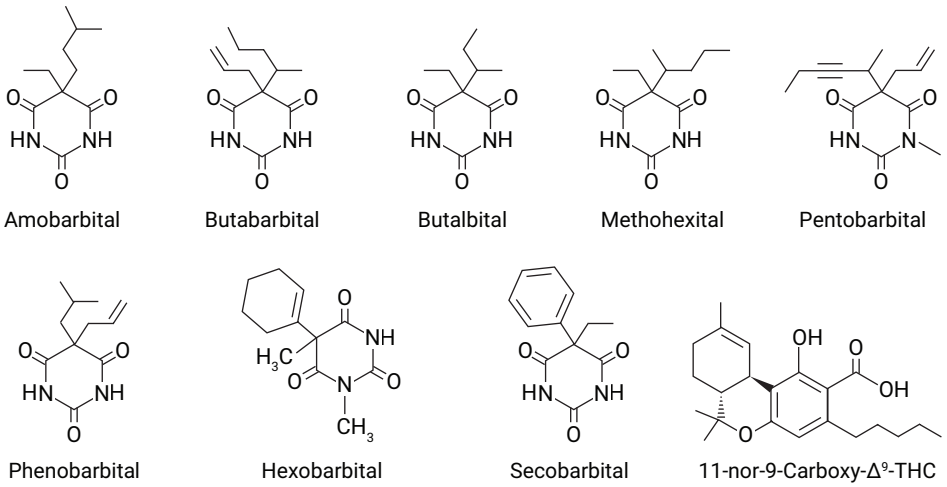

Figure 1. Analyte structures.

Experimental

LC Configuration and parameters

Configuration		
Pump	Two Agilent 1290 Infinity II binary pumps (p/n G7120A)	
Multisampler	Agilent 1290 Infinity II multisampler (p/n G7167A)	
Column compartment	Agilent 1290 Infinity thermostatted column compartment with a 2-position 10 port valve (p/n G7116B)	
Needle wash	50:20:20:10 IPA:MeOH:ACN:H_2O	
Autosampler temperature	10 °C	
Injection volume	10 µL	
Analytical column	2 Agilent Poroshell 120 EC-C18, 2.1 × 100 mm, 1.9 µm, LC columns (p/n 695775-902)	
Column temperature	55 °C	
Mobile phase A	5 mM Ammonium acetate in water	
Mobile phase B	Acetonitrile	
Flow rate	0.35 mL/min	
Gradient	**Eluent pump** Time (min) %B 0.00 35 1.60 45 1.61 98 3.00 98 3.01 45 3.59 45	**Regeneration pump** Time (min) %B 0.00 98 1.40 98 1.50 35
Stop time	Eluent pump: 3.65 minutes	Regeneration pump: no limit
Valve position	0.00 minutes Current position 3.59 minutes Next position	

Chemicals and reagents

Human urine used for matrix-matched calibrators was purchased from Golden West Biologicals, Inc, (Temecula, CA). Standards and internal standards were bought from Cerillant Corporation (Round Rock, TX). Sample preparation and LC solvents were acquired from Honeywell (Muskegon, MI).

Sample preparation

Standards were spiked into drug-free human urine solution (10 %). The calibration curve was created by serial dilution following a pattern of 1:2:2:2.5. Concentrations ranged from 0.5 to 5,000 ng/mL. Internal standards were added to a final concentration of 200 ng/mL. Then, 10 µL were injected onto the LC/MS system.

Data analysis

Data acquisition was performed using Agilent MassHunter Acquisition Software (B.08.00). MS/MS transitions were obtained using Agilent MassHunter Acquisition optimizer software to determine optimal parent and product ions, fragmentor voltages, and collision energies. Data were analyzed using Agilent MassHunter Quantitative Analysis Software (B.08.00) and Qualitative Analysis Software (B.07.00).

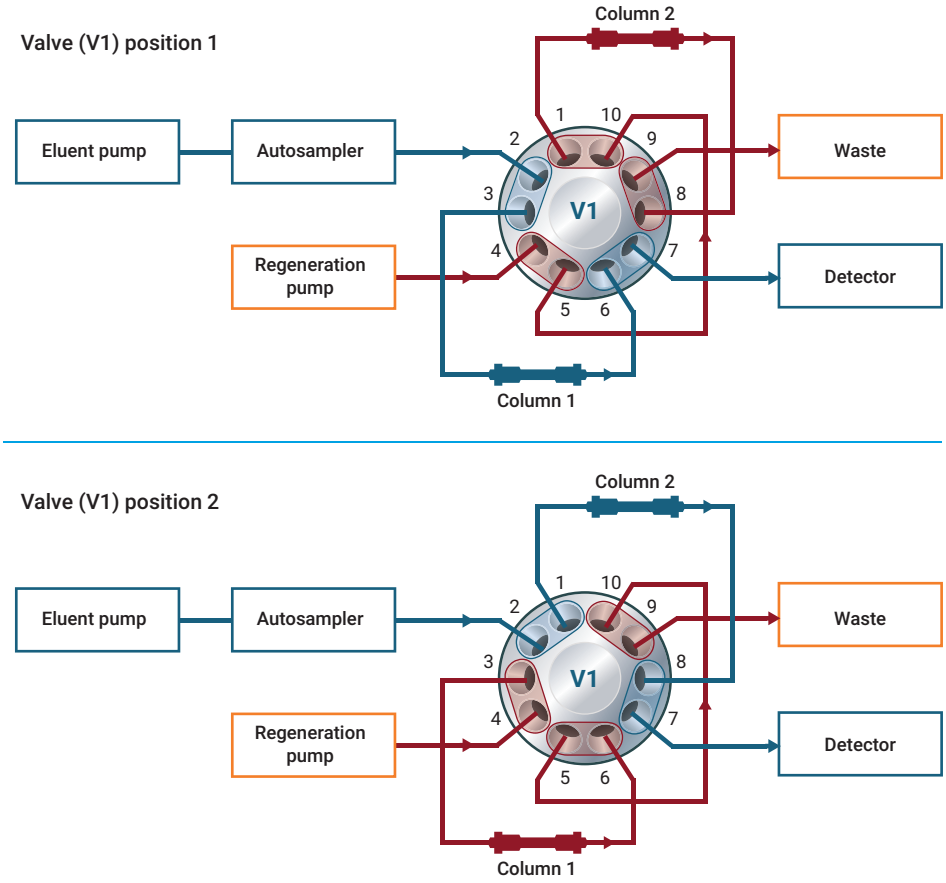

Figure 2. Alternating column regeneration (ACR) valve configuration.

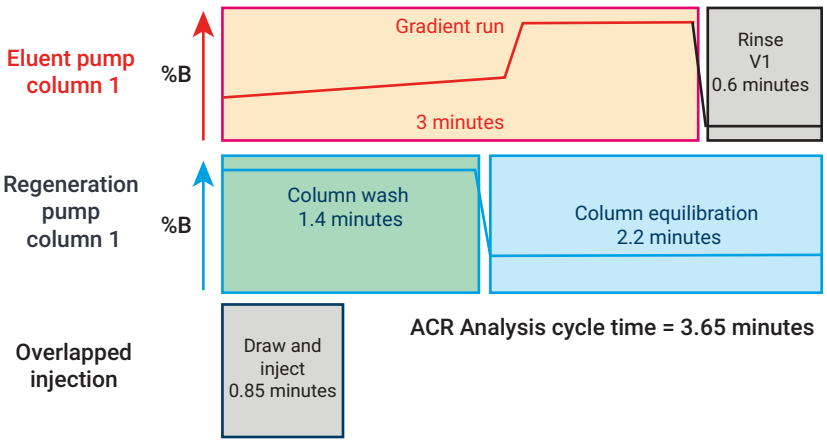

Figure 3. Graphical timeline for ACR analysis.

Results and discussion

Chromatography

The main emphasis of this work was increased throughput. Therefore, under these chromatographic conditions, the isobars amobarbital and pentobarbital did not separate, and are reported as a single peak (Figure 4). If separation between amobarbital and pentobarbital is required, adjust the gradient and the run time to achieve baseline separation between those isobars, as shown in Figure 6.

LC/TQ Mass spectrometer configuration and parameters

Configuration	
Instrument	Agilent 6470 triple quadrupole mass spectrometer with Agilent Jet Stream
MS/MS mode	MRM
Ionization mode	Negative
Drying gas temperature	150 °C
Drying gas flow	11 L/min
Nebulizer pressure	30 psi
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Nozzle voltage	2,000 V
Capillary voltage	6,000 V
Delta EMV	800 V
Q1/Q2 resolution	Unit/Unit
Dwell time	50 ms
Cell accelerator voltage	4 V

MS/MS Compound information for analytes and internal standards

Compound	ISTD?	Precursor ion (*m/z*)	Product ion (*m/z*)	RT (min)	Fragmentor (V)	Collision energy (V)
Amo/Pentobarbital		225.1	182	1.78	105	12
Amo/Pentobarbital		225.1	42	1.78	105	24
AmoPentobarbital-D5	✓	230.1	42	1.78	105	24
Butabarbital		211.1	168	1.32	165	12
Butabarbital		211.1	42	1.32	165	40
Butalbital		223.1	180	1.45	165	8
Butalbital		223.1	42	1.45	165	36
Butalbital-D5	✓	228.1	42	1.45	165	36
Hexobarbital		235.1	42	1.85	85	20
Methohexital		261.1	42	2.66	65	20
Phenobarbital		231.1	188	1.17	140	8
Phenobarbital		231.1	42	1.17	140	36
Phenobarbital-D5	✓	236.1	42	1.17	140	36
Secobarbital		237.1	194	2.02	170	12
Secobarbital		237.1	42	2.02	170	36
Secobarbital-D5	✓	242.1	42	2.02	170	36
THC-A		343.2	299.2	2.88	125	24
THC-A		343.2	245	2.88	125	36
THC-A-D9	✓	352.2	254.1	2.88	125	32

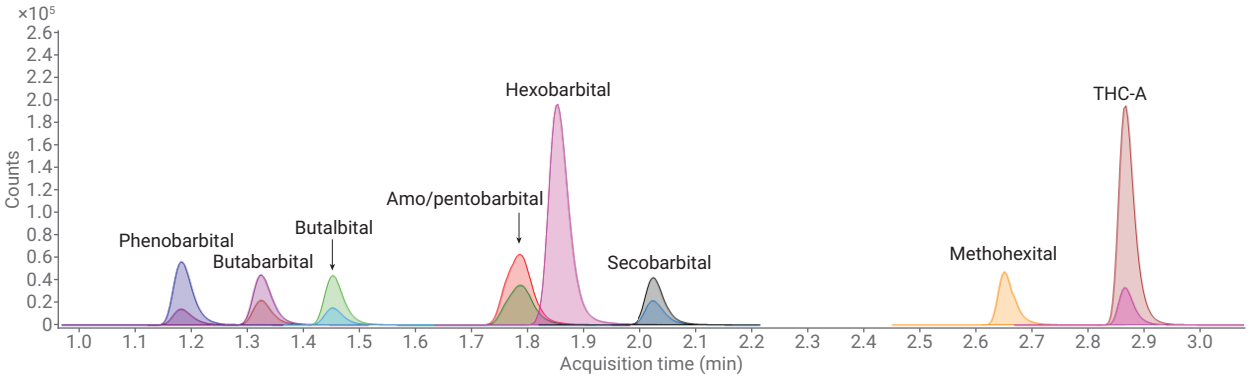

Figure 4. dMRM Chromatogram showing elution of the nine compounds at 500 ng/mL.

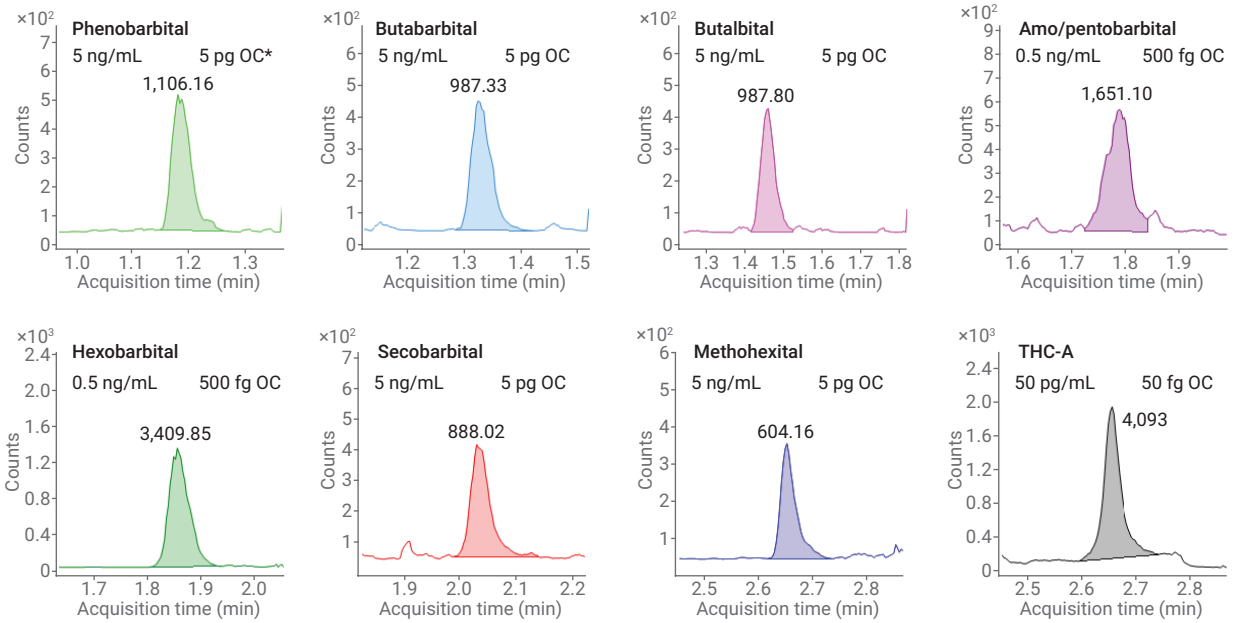

Figure 5. Chromatograms at lower limit of quantitation (LLOQ).

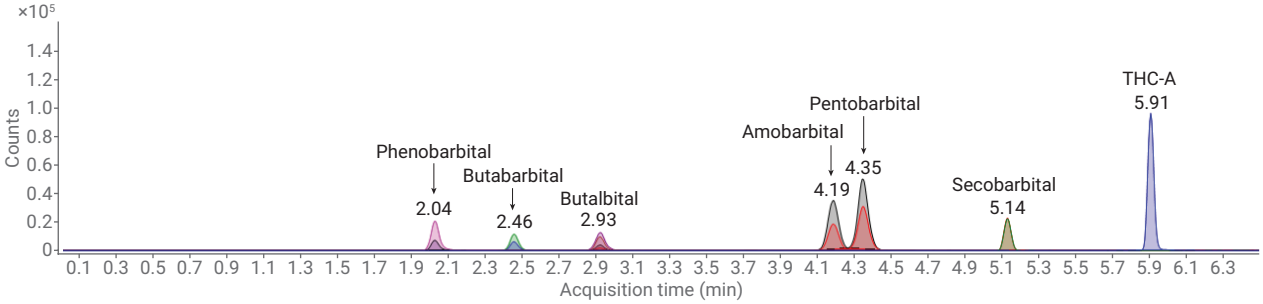

Figure 6. Chromatogram with amobarbital and pentobarbital baseline separation.

Calibration curves

All calibration curves were linear, and a 1/x weighting factor was used. Figure 7 shows examples of calibration curves, and Table 1 lists curve fit correlations (R^2). For better visual presentation, amo/pentobarbital and THC-A are shown with logarithmic scales so that all calibration points can be displayed.

Table 1. Linear curve correlation coefficients.

Name	Transition	R^2
Amo/Pentobarbital	225.1 → 42.0	0.9963
Butabarbital	211.1 → 42.0	0.9980
Butalbital	223.1 → 42.0	0.9982
Hexobarbital	235.1 → 42.0	0.9928
Methohexital	261.1 → 42.0	0.9916
Phenobarbital	231.1 → 42.0	0.9968
Secobarbital	237.1 → 42.0	0.9974
THC-A	343.2 → 299.2	0.9992

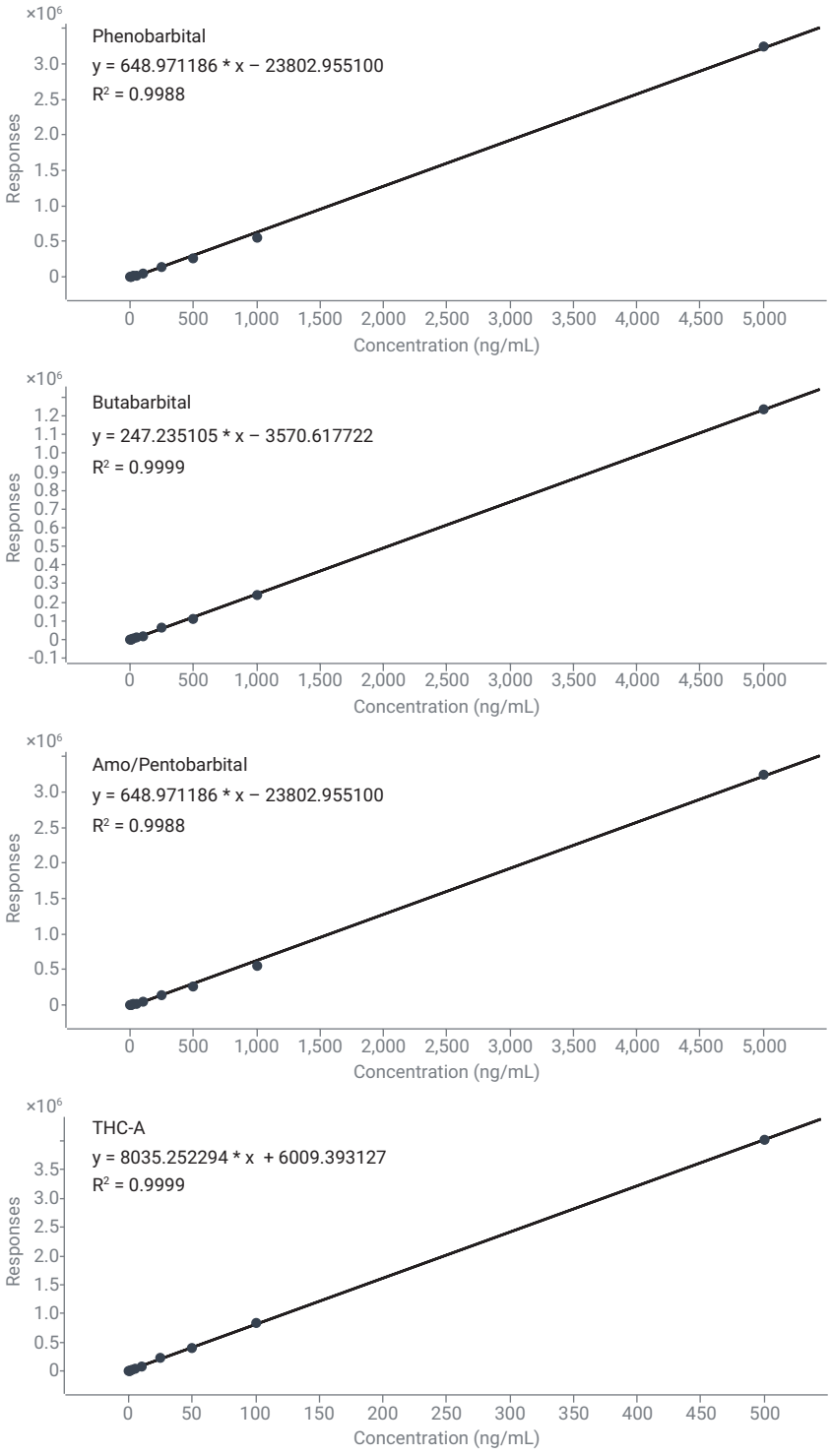

Figure 6. Example calibration curves.

Quantitation results

Table 2 shows all quantitation results. This was a 10-point calibration curve ranging from 5 ng/mL to 5,000 ng/mL for all compounds except amo/pentobarbital and THC-A, which ranged from 0.5 ng/mL to 500 ng/mL. All compounds were analyzed down to 1 ng/mL (0.1 ng/mL for amo/pentobarbital and THC-A), and showed a signal-to-noise ratio of 5 or better.

Table 2. Quantitation results.

Level	Conc. (ng/mL)	Phenobarbital results			Butabarbital results			Butalbital results			Amo/Pentobarbital results		
		RT	Final conc.	Resp.	RT	Final conc.	Resp.	RT	Final conc.	Resp.	RT	Final conc.	Resp.
1	2.5/0.25	1.19	2.68	326	1.33	3.84	558	1.46	3.44	381	1.78	0.24	1155
2	5/0.5	1.18	5.21	1107	1.32	5.60	987	1.46	6.00	988	1.79	0.59	1971
3	10/1	1.19	8.86	2230	1.33	8.96	1802	1.46	8.99	1696	1.78	0.96	3911
4	25/2.5	1.18	23.92	6875	1.33	23.70	5376	1.46	23.48	5129	1.79	2.18	11737
5	50/5	1.18	40.24	11906	1.33	40.86	9540	1.46	39.96	9035	1.79	3.71	21176
6	100/10	1.18	80.44	24298	1.33	74.78	17770	1.46	78.94	18270	1.79	9.20	62732
7	250/25	1.19	255.89	78389	1.33	254.05	61268	1.46	256.42	60317	1.79	23.49	140534
8	500/50	1.18	454.96	139761	1.33	461.05	111491	1.45	464.14	109530	1.79	42.42	257751
9	1000/100	1.18	994.75	306175	1.33	979.64	237317	1.46	1041.56	246330	1.79	88.96	545678
10	5000/500	1.18	5074.33	1563889	1.33	5090.02	1234614	1.46	5019.58	1188795	1.79	523.99	3240808

Level	Conc. (ng/mL)	Hexobarbital results			Secobarbital results			Methohexital results			THC-A results		
		RT	Final conc.	Resp.	RT	Final conc.	Resp.	RT	Final conc.	Resp.	RT	Final conc.	Resp.
1	2.5/0.25	1.86	3.81	1777	2.03	3.73	365	2.65	2.51	391	2.87	0.21	2471
2	5/0.5	1.85	6.11	3410	2.03	6.02	885	2.65	5.85	604	2.87	0.50	4773
3	10/1	1.85	9.35	7489	2.03	8.98	1559	2.65	9.60	1554	2.87	0.98	8687
4	25/2.5	1.86	19.74	20576	2.04	22.80	4702	2.65	21.59	4594	2.87	2.81	23554
5	50/5	1.86	34.93	39720	2.03	36.86	7898	2.65	33.59	7637	2.87	4.80	39706
6	100/10	1.86	71.48	85748	2.03	79.09	17502	2.65	69.44	16727	2.87	9.87	80784
7	250/25	1.86	230.17	285638	2.03	239.81	54046	2.65	223.89	55890	2.87	27.89	226961
8	500/50	1.85	409.39	511383	2.03	464.13	105055	2.65	399.36	100383	2.87	48.75	396146
9	1000/100	1.85	931.90	1169527	2.03	1076.06	244202	2.65	883.23	223071	2.87	103.10	836973
10	5000/500	1.86	5224.62	6576592	2.03	5005.01	1137604	2.65	5290.95	1340690	2.87	495.35	4018515

Conclusions

This work combined detection of barbiturates and THC-A into a single analytical method. More emphasis was placed on increasing throughput using alternating column regeneration (ACR). To have a fast analytical method, there was no separation between the isobars amobarbital and pentobarbital, and the initial analysis time for all compounds was 5.0 minutes. The addition of ACR reduced the analysis runtime to 3.7 minutes injection to injection, which translates to a 26 % improvement in throughput. Calibration curves for all compounds were linear, with correlations of 0.99 or better. LLOQs for urine-spiked phenobarbital, butalbital, butabarbital, secobarbital, and methohexital were at least 5.0 ng/mL or better, and for amo/pentobarbital and THC-A the LLOQs were 0.5 ng/mL or better.

Assessing potential interferences for this analytical method of urine matrices from different suppliers prepared for LC/MS analysis through a range of more sample preparation techniques is a project for the future.

References

1. Workman, H.; *et al.* A Combined Method for the Analysis of Barbiturates and 11-nor-9-carboxy Δ^9THC in Urine by LC/MS/MS. *SOFT* **2011**, Poster.
2. Cichelli, J.; Doyle, R. M. LC/MS/MS Analysis of Barbiturates in Urine, Oral Fluid and Blood. *MSACL* **2015**, Poster.

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