Simultaneous Quantitation of 43 Drugs in Human Urine with a "Dilute-and-Shoot" LC-MS/MS Method

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Application Note 576

Key Words

TSQ Quantum Access MAX, forensic toxicology, drugs of abuse, pain management drugs, urine, quantitation

Goal

The goal of this work was to develop a simple "dilute-and-shoot" liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantitation of 43 drugs of abuse, including pain management drugs, in human urine for forensic toxicology purposes. The drugs to be analyzed included opioids, amphetamines, benzodiazepines, cocaine, buprenorphine, methadone, and some of their metabolites. An additional objective was to use ultra-high-pressure liquid chromatography (UHPLC) to improve throughput and sensitivity of the method.

Introduction

LC-MS/MS has become more accepted as the tool for quantitative analysis of drugs in forensic toxicology laboratories. This technique enables simultaneous detection of multiple analytes of interests and is compatible with a simple "dilute-and-shoot" sample preparation method for urine samples.

Methods

Sample Preparation

Nine individual human urine and pure water samples were spiked with 20 and 200 ng/mL of the 43 drugs of abuse, pain management drugs, and with internal standards (IS). The samples were then mixed with β-glucuronidase and incubated at 60 °C for hydrolysis. Methanol was added to the mixture and the supernatant was diluted with water. The final dilution factor was 20. The mixture was centrifuged at 17,000 *g* for 5 minutes. Fifty microliter injections of the supernatant were analyzed by LC-MS/MS.

Blank human urine was used as the matrix for calibration samples. The concentrations of the calibrators were 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL. Concentration of the internal standards in all samples was 250 ng/mL.

LC-MS/MS Conditions

LC-MS/MS analysis was performed on a Thermo Scientific™ Accela™ 1250 pump and Accela Open autosampler coupled to a Thermo Scientific TSQ Quantum Access MAX™ triple stage quadrupole mass spectrometer. The analytical column was a Thermo Scientific Accucore™ PFP column (50 × 2.1 mm, 2.6 µm particle size) maintained at room temperature. Details of the LC gradient and mobile phases (MP) are as follows:

Time (min)	Flow rate (mL/min)	Gradient	MPA (%)	MPB (%)	MPC (%)
0.00	0.75	Step	95	5	0
0.50	0.75	Ramp	60	40	0
2.60	0.75	Ramp	5	95	0
4.50	1.00	Step	0	100	0
5.50	1.00	Step	0	0	100
5.75	1.00	Step	95	5	0

MPA: 10 mM NH_4Ac and 0.1% formic acid in water
MPB: 10 mM NH_4Ac and 0.1% formic acid in methanol
MPC: acetonitrile/isopropanol/acetone 9:9:2 (v/v/v)

The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. The MS conditions were as follows:

Spray voltage (V)	4000
Vaporizer temperature (°C)	300
Sheath gas pressure (arbitrary units)	50
Auxiliary gas pressure (arbitrary units)	15
Capillary temperature (°C)	300

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Data were acquired in selected-reaction monitoring (SRM) mode. SRM transitions for the 43 drugs and their internal standards are shown in Table 1. For each analyte and internal standard, two SRM transitions were monitored. One of transition was used as the quantifier and the other as the qualifier. The signal ratio between the qualifier and the quantifier was used to evaluate the validity of the results.

Validation

The validation procedure included tests for the following: 1) matrix effects; 2) lower limit of quantitation (LLOQ), linear range, accuracy, and precision; and 3) carryover.

Table 1. Drug analytes, their corresponding internal standards, and the SRM transitions for both analytes and internal standards

Analyte	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)	Ion Ratio (%)	Corresponding Internal Standard	Precursor Ion (*m/z*)	Quantifier Ion (*m/z*)	Qualifier Ion (*m/z*)
6-MAM	328.10	165.10	211.00	86.0	6-MAM-d3	331.20	165.10	211.10
7-Amino-clonazepam	286.00	222.10	250.10	95.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
7-Amino-flunitrazepam	284.10	135.10	226.10	52.0	7-Amino-flunitrazepam-d7	291.11	138.20	230.10
7-Aminonitrazepam	252.10	121.10	224.10	16.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
α-Hydroxy-alprazolam	325.10	216.10	297.10	52.0	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
Alprazolam	309.40	205.00	281.00	76.0	Temazepam-d5	306.10	260.10	288.10
Amphetamine	136.10	65.30	91.20	10.0	Amphetamine-d5	141.10	92.20	93.20
Benzoylecgonine	290.10	105.10	168.10	30.0	Benzoylecgonine-d3	293.10	105.10	171.10
Benzylpiperazine	177.10	65.30	91.20	16.0	Benzylpiperazine-d7	184.10	70.20	98.20
Buprenorphine	468.30	396.30	414.30	120.0	Diazepam-d5	290.12	198.10	227.10
Carisprodol	261.20	62.10	97.10	58.5	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
Clonazepam	315.90	214.00	270.00	26.6	Temazepam-d5	306.10	260.10	288.10
Cocaine	304.10	82.20	182.10	17.0	Amphetamine-d5	141.10	92.20	93.20
Codeine	300.10	165.00	215.00	91.0	Codeine-d3	303.20	165.10	215.10
Diazepam	285.10	193.10	222.10	72.0	Diazepam-d5	290.12	198.10	227.10
EDDP	279.20	235.20	250.20	54.5	Temazepam-d5	306.10	260.10	288.10
Fentanyl	337.20	105.20	188.20	67.0	Temazepam-d5	306.10	260.10	288.10
Flunitrazepam	314.40	239.10	268.10	32.5	Temazepam-d5	306.10	260.10	288.10
Flurazepam	388.10	288.10	315.10	11.5	Temazepam-d5	306.10	260.10	288.10
Hydrocodone	300.20	171.00	199.00	34.5	MDA-d5	185.10	110.20	137.10
Hydromorphone	286.11	157.10	185.10	64.0	Benzylpiperazine-d7	184.10	70.20	98.20
Lorazepam	321.00	275.00	303.00	64.0	α-Hydroxy-alprazolam-d5	330.10	221.10	302.10
MDA	180.10	105.20	135.10	79.0	MDA-d5	185.10	110.20	137.10
MDEA	208.10	135.10	163.00	24.0	Nordiazepam-d5	276.10	165.00	213.10
MDMA	194.10	135.10	163.10	40.0	MDMA-d5	199.10	135.10	165.10
Meperidine	248.20	174.20	220.10	28.0	Diazepam-d5	290.12	198.10	227.10
Methadone	310.20	105.10	265.10	29.0	Diazepam-d5	290.12	198.10	227.10
Methamphetamine	150.10	65.30	91.20	9.5	Methamphetamine-d5	155.10	91.20	92.20
Midazolam	326.10	249.20	291.20	28.0	Diazepam-d5	290.12	198.10	227.10
Morphine	286.10	152.10	165.00	78.0	Morphine-d3	289.10	152.10	165.10
Naloxone	328.21	212.00	310.10	23.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
Naltrexone	342.20	270.10	324.20	16.0	MDA-d5	185.10	110.20	137.10
Norbuprenorphine	414.30	187.10	340.30	99.0	Temazepam-d5	306.10	260.10	288.10
Nordiazepam	271.00	140.10	208.10	100.5	Nordiazepam-d5	276.10	165.00	213.10
Norfentanyl	233.20	55.30	84.30	16.0	MDMA-d5	199.10	135.10	165.10
Normeperidine	234.20	111.10	160.10	0.3	Temazepam-d5	306.10	260.10	288.10
Oxazepam	287.00	241.00	269.00	82.0	Oxazepam-d5	292.10	246.10	274.10
Oxycodone	316.20	241.20	298.20	22.5	Benzoylecgonine-d3	293.10	105.10	171.10
Oxymorphone	302.10	227.10	284.20	35.0	7-Amino-clonazepam-d4	290.11	226.10	254.10
PCP	244.20	86.20	159.10	84.5	Diazepam-d5	290.12	198.10	227.10
Propoxyphene	340.20	58.20	91.10	15.0	Diazepam-d5	290.12	198.10	227.10
Temazepam	301.00	255.00	283.00	36.0	Temazepam-d5	306.10	260.10	288.10
Tramadol	264.20	58.30	246.10	3.0	Temazepam-d5	306.10	260.10	288.10

Results and Discussion

Matrix Effects

Matrix effects were assessed with the nine individual human urine samples. Absolute recovery was determined by comparing the signals of unlabeled drugs in urine and water samples. Relative recovery was determined by comparing the analyte/IS ratio in urine and water samples. The recovery/matrix effects results are summarized in Table 2. All 43 drugs had almost full absolute recovery (between 80% and 120%), except morphine for which the matrix effect was compensated by the use of its internal standard, morphine-d3. The observed precision from the nine individual human urine samples was below 15% for most of the 43 drugs.

Table 2. Summary of matrix effects

Drug	Average Absolute Recovery (%, n=9)		CV (%, n=9)		Average Relative Recovery (%, n=9)		CV (%, n=9)	
	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL	20 ng/mL	200 ng/mL
6-MAM	86.7	92.3	16.2	12.2	95.1	100.2	5.6	5.7
7-Amino-clonazepam	96.4	108.4	11.0	11.6	90.3	103.7	6.1	5.8
7-Amino-flunitrazepam	86.8	90.5	11.4	8.4	97.1	102.1	6.3	5.1
7-Aminonitrazepam	86.0	85.5	12.3	9.6	80.6	81.9	9.9	8.2
α-Hydroxy-alprazolam	87.4	87.4	12.9	6.7	99.4	96.2	10.4	4.1
Alprazolam	94.0	89.1	26.0	16.6	95.1	84.8	22.9	13.9
Amphetamine	109.5	112.3	11.8	8.0	112.4	110.8	16.7	3.8
Benzoylecgonine	82.7	85.7	13.0	12.7	98.7	100.9	4.6	3.6
Benzylpiperazine	87.3	85.4	10.0	7.2	100.4	100.6	8.7	7.5
Buprenorphine	108.4	96.9	15.0	6.2	118.1	97.2	14.6	5.4
Carisprodol	88.0	96.3	13.1	11.0	100.5	105.8	13.6	8.4
Clonazepam	100.7	98.4	9.5	6.9	103.4	94.4	13.6	9.5
Cocaine	93.6	93.5	7.4	8.2	95.5	92.2	5.3	5.0
Codeine	93.9	98.9	8.6	8.2	99.3	98.0	3.3	7.2
Diazepam	98.0	96.6	14.0	9.1	106.5	96.7	11.7	6.5
EDDP	103.8	99.2	6.8	2.9	106.8	95.0	13.7	6.2
Fentanyl	98.6	100.9	4.1	2.8	101.4	96.7	10.7	5.8
Flunitrazepam	85.7	86.9	18.8	14.7	87.1	82.9	14.6	12.1
Flurazepam	97.5	103.1	4.2	3.9	100.2	98.8	11.7	5.8
Hydrocodone	91.5	96.4	15.1	13.5	95.2	97.9	7.8	9.6
Hydromorphone	91.2	94.5	11.0	10.4	104.6	110.8	7.2	5.4
Lorazepam	105.7	90.5	16.5	6.0	120.7	99.7	17.2	5.7
MDA	96.6	105.8	16.1	9.6	100.6	107.9	8.7	6.4
MDEA	95.6	94.0	11.8	10.1	99.0	82.8	9.7	10.6
MDMA	92.3	94.3	9.3	7.8	106.1	102.4	2.4	4.1
Meperidine	88.4	88.4	9.8	9.9	96.2	88.5	7.4	7.8
Methadone	101.6	103.2	3.2	3.4	111.1	103.6	8.9	5.4
Methamphetamine	94.6	86.2	12.3	11.1	105.5	94.1	8.4	5.6
Midazolam	98.4	97.4	9.5	5.5	107.1	97.6	6.0	3.5
Morphine	48.1	53.8	6.0	8.2	90.5	98.4	6.9	5.4
Naloxone	124.2	129.4	17.9	16.1	116.1	123.5	9.9	7.7
Naltrexone	96.1	100.2	12.6	10.9	100.3	101.9	5.2	6.1
Norbuprenorphine	76.9	104.6	19.4	14.2	78.9	99.9	20.4	11.6
Nordiazepam	102.8	107.1	21.3	7.3	106.3	94.2	19.8	7.5
Norfentanyl	89.5	92.2	11.4	8.2	103.2	100.1	12.4	4.5
Normeperidine	81.7	92.0	11.9	11.6	83.1	87.7	7.8	8.4
Oxazepam	93.8	91.3	10.8	5.1	113.4	102.1	6.8	4.7
Oxycodone	80.4	84.7	8.8	10.9	97.0	100.1	9.7	6.1
Oxymorphone	107.0	101.4	15.2	12.9	100.1	97.0	8.2	9.0
PCP	100.8	100.5	4.2	4.3	110.3	100.9	8.8	5.1
Propoxyphene	101.3	103.8	6.8	5.8	111.2	104.1	15.3	4.4
Temazepam	95.3	102.2	14.2	7.1	97.3	97.7	12.3	4.6
Tramadol	77.8	84.8	14.1	12.9	78.9	80.8	10.1	10.4

Lower Limit of Quantitation, Linear Range, Accuracy, and Precision

The LLOQ of these 43 drugs and other aspects of analytical performances of this method are summarized in Table 3. Linear fit with 1/X weighting was used for calibration curves of all the 43 drugs. The LLOQ for these 43 drugs was determined to be between 2 and 20 ng/mL except for tramadol, which was 50 ng/mL. At the LLOQ, the accuracy ranged between 89.9% and 118.4%, and precision ranged between 3.6% and 19.5%. The method was linear to 1000 ng/mL for all the drugs. Figure 1 shows the calibration curves of six typical pain management drugs in human urine.

Table 3. Lower limit of quantitation, linear range, accuracy, and precision

Drug	Retention Time (min)	LLOQ (ng/mL)	Accuracy at LLOQ (%, n=4)	CV at LLOQ (%, n=4)	Linear Range (ng/mL)	R^2	Precision 20 ng/mL (%, n=6)	Precision 200 ng/mL (%, n=6)
6-MAM	2.97	2	95.0	14.6	2–1000	0.9955	5.8	2.8
7-Amino-clonazepam	2.76	5	95.1	10.1	5–1000	0.9988	3.4	4.0
7-Amino-flunitrazepam	3.31	5	101.0	13.7	5–1000	0.9980	5.3	4.0
7-Aminonitrazepam	2.51	2	102.0	9.6	2–1000	0.9972	3.7	3.2
α-Hydroxy-alprazolam	3.87	20	94.0	10.0	20–1000	0.9972	6.9	5.9
Alprazolam	4.11	5	94.1	13.1	5–1000	0.9950	2.7	1.2
Amphetamine	2.97	20	94.9	7.7	20–1000	0.9944	5.4	5.5
Benzoylecgonine	2.99	5	92.3	3.6	5–1000	0.9990	2.7	2.0
Benzylpiperazine	2.70	10	96.0	10.4	10–1000	0.9979	9.5	5.0
Buprenorphine	4.50	20	94.7	17.3	20–1000	0.9976	6.0	6.1
Carisprodol	3.80	10	104.5	11.3	10–1000	0.9903	9.5	6.3
Clonazepam	4.00	20	92.7	7.7	20–1000	0.9954	9.1	6.1
Cocaine	4.23	5	101.2	7.4	5–1000	0.9969	4.0	3.7
Codeine	2.82	10	110.4	18.3	10–1000	0.9978	6.6	3.8
Diazepam	4.24	5	93.0	11.9	5–1000	0.9979	7.0	3.4
EDDP	4.90	10	106.5	3.9	10–1000	0.9944	4.5	2.2
Fentanyl	4.62	2	108.9	3.7	2–1000	0.9975	4.8	1.8
Flunitrazepam	4.12	20	93.7	17.2	20–1000	0.9904	6.4	4.4
Flurazepam	4.57	2	118.4	3.6	2–1000	0.9961	4.9	2.4
Hydrocodone	3.16	2	106.6	9.6	2–1000	0.9988	7.8	2.8
Hydromorphone	2.25	2	89.9	13.2	2–1000	0.9979	8.2	3.1
Lorazepam	3.86	20	92.5	17.3	20–1000	0.9943	2.2	9.6
MDA	3.16	10	93.1	6.8	10–1000	0.9974	1.1	3.1
MDEA	3.97	2	104.3	4.5	2–1000	0.9937	7.5	4.4
MDMA	3.61	5	97.3	4.3	5–1000	0.9975	7.6	2.2
Meperidine	4.20	5	101.2	9.6	5–1000	0.9986	5.5	4.6
Methadone	4.95	5	100.3	3.8	5–1000	0.9982	4.2	3.0
Methamphetamine	3.51	5	106.0	5.1	5–1000	0.9979	5.0	4.0
Midazolam	4.48	2	117.1	12.7	2–1000	0.9983	7.0	4.3
Morphine	1.71	5	93.0	13.6	5–1000	0.9990	5.0	3.3
Naloxone	2.86	10	102.3	10.9	10–1000	0.9944	3.3	2.9
Naltrexone	3.11	5	101.9	7.0	5–1000	0.9985	5.1	1.6
Norbuprenorphine	4.13	20	101.4	14.4	20–1000	0.9955	3.9	8.4
Nordiazepam	4.06	10	97.1	19.5	10–1000	0.9948	8.4	3.8
Norfentanyl	3.68	10	102.5	6.3	10–1000	0.9985	7.1	2.3
Normeperidine	4.00	2	116.2	11.2	2–1000	0.9982	7.3	4.2
Oxazepam	3.88	20	108.0	15.0	20–1000	0.9970	10.9	6.4
Oxycodone	3.03	5	91.9	11.7	5–1000	0.9982	2.6	2.3
Oxymorphone	2.01	2	93.3	9.5	2–1000	0.9946	10.0	2.6
PCP	4.83	2	100.9	4.0	2–1000	0.9981	7.8	3.0
Propoxyphene	4.70	10	113.6	4.1	10–1000	0.9978	7.3	5.2
Temazepam	4.05	5	104.6	16.9	5–1000	0.9981	5.6	2.2
Tramadol	4.04	50	98.8	2.5	50–1000	0.9970	NA	2.5

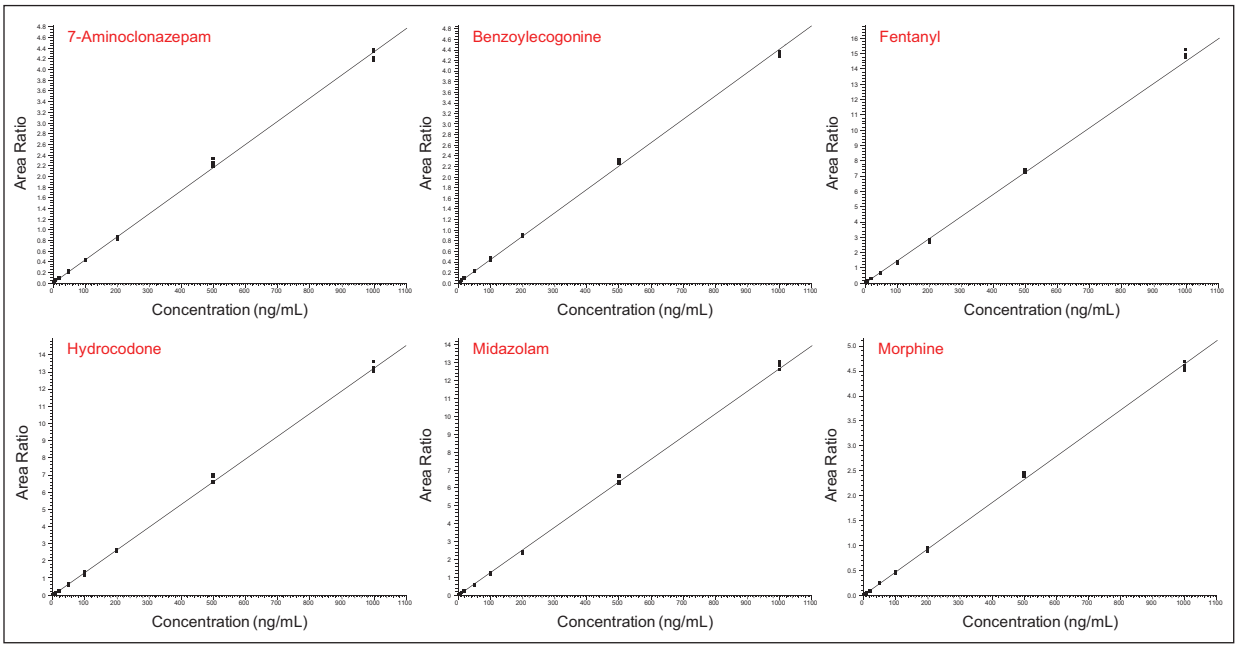

Figure 1. Calibration curves of six selected drugs in spiked human urine

Method precision was also assessed with spiked human urine samples at low and high quality control (QC) concentrations of 20 and 200 ng/mL, respectively (Table 2). Precision values at low (20 ng/mL) and high (200 ng/mL) quality control concentrations ranged between 1.1% and 10.9% (Table 2). Figure 2 shows both the quantifier and qualifier SRM chromatograms of 20 selected pain management drugs spiked at 20 ng/mL in human urine.

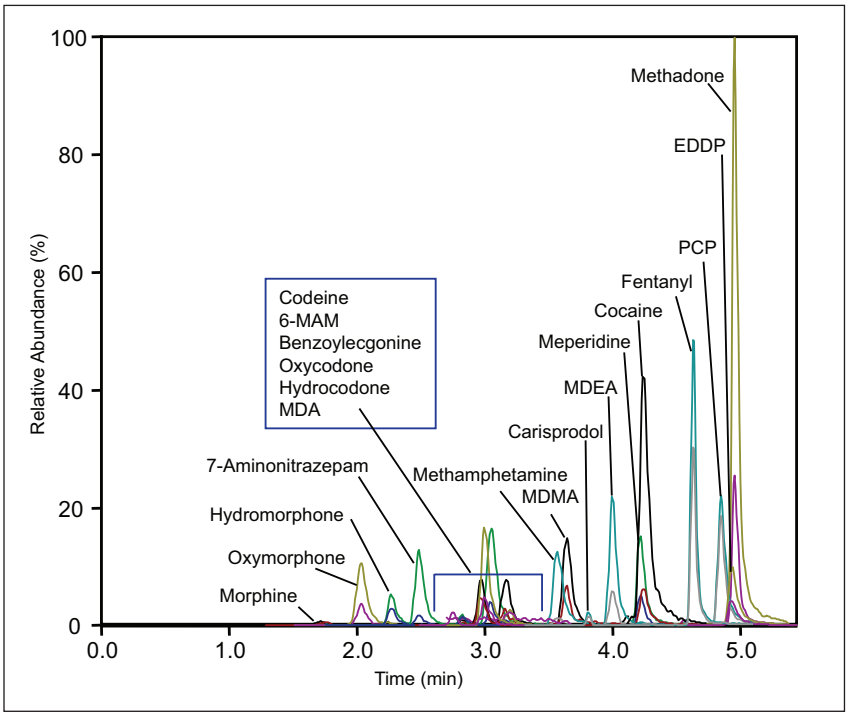

Figure 2. SRM chromatograms of 20 selected drugs at 20 ng/mL in spiked human urine

Carryover

The lowest calibrator was analyzed after the highest calibrator. No carryover causing elevated measurements of the drugs in the lowest calibrator was observed.

Conclusion

The developed method provides a simple, fast, and sensitive way for forensic toxicology labs to simultaneously quantify 43 drugs of abuse, including pain management drugs, in human urine by LC-MS/MS. The method provided LLOQ values of 2–20 ng/mL for 42 of the 43 drugs, and was linear to 1000 ng/mL. Minimal ion suppression and no carryover were observed in matrix samples. At the LLOQ, the accuracy ranged between 89.9% and 118.4%. Method precision ranged between 1.1% and 10.9% at low and high QC samples.

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