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Clinical research and toxicology

Quantitation of 22 fentanyl analog compounds with a TSQ Altis MD mass spectrometer

Authors

Stephanie Samra and Richard Gibson

Thermo Fisher Scientific, San Jose, CA

Keywords

Fentanyl, fentanyl analog, TSQ Altis MD mass spectrometer, Vanquish MD HPLC system, TraceFinder software, Accucore HPLC column, reversed-phase UHPLC, toxicology, Opioid Certified Reference Material Kit, CDC, TOMS, quantitation, drugs of abuse, synthetic opioids

Goal

Demonstrate the speed and sensitivity of the Thermo Scientific™ TSQ Altis™ MD Triple Quadrupole mass spectrometer for fast, accurate analysis of 22 fentanyl-related compounds, including fentanyl, in urine for clinical research and toxicology.

Introduction

The United States is facing an opioid crisis that includes not only the abuse of prescription drugs, but also synthetic opioids. According to the Centers for Disease Control and Prevention (CDC), rates of overdose deaths involving synthetic opioids (other than methadone) increased by 30% from 2019 to 2020 and another 15% in 2021[1]. More than 71,000 people died from synthetic opioids (primarily involving fentanyl) in 2021 alone and accounted for over 88% of all opioid related deaths. The drastic rise in drug use has created a demand for toxicology labs to test for these compounds. To address the need, the CDC, in collaboration with Cerilliant™ Corporation, released a Traceable Opioid Material™ (TOM) kit consisting of 22 fentanyl analog compounds with matched ^{13}C and ^{15}N isotopically labeled internal standards for quantitation and confident identification. Herein, we present a method for quantitation of the TOM kit compounds in urine that includes sample preparation by supported liquid extraction (SLE) and quantitation by selected reaction monitoring (SRM) on a Thermo Scientific™ TSQ Altis™ MD Triple Quadrupole mass spectrometer.

Experimental

Target analytes

A panel consisting of 22 fentanyl analogs was analyzed and quantitated by internal standard calibration. The chemical structures of these compounds are presented in Figure 1. Each fentanyl analog had its own isotopically labeled internal standard as listed in Table 4.

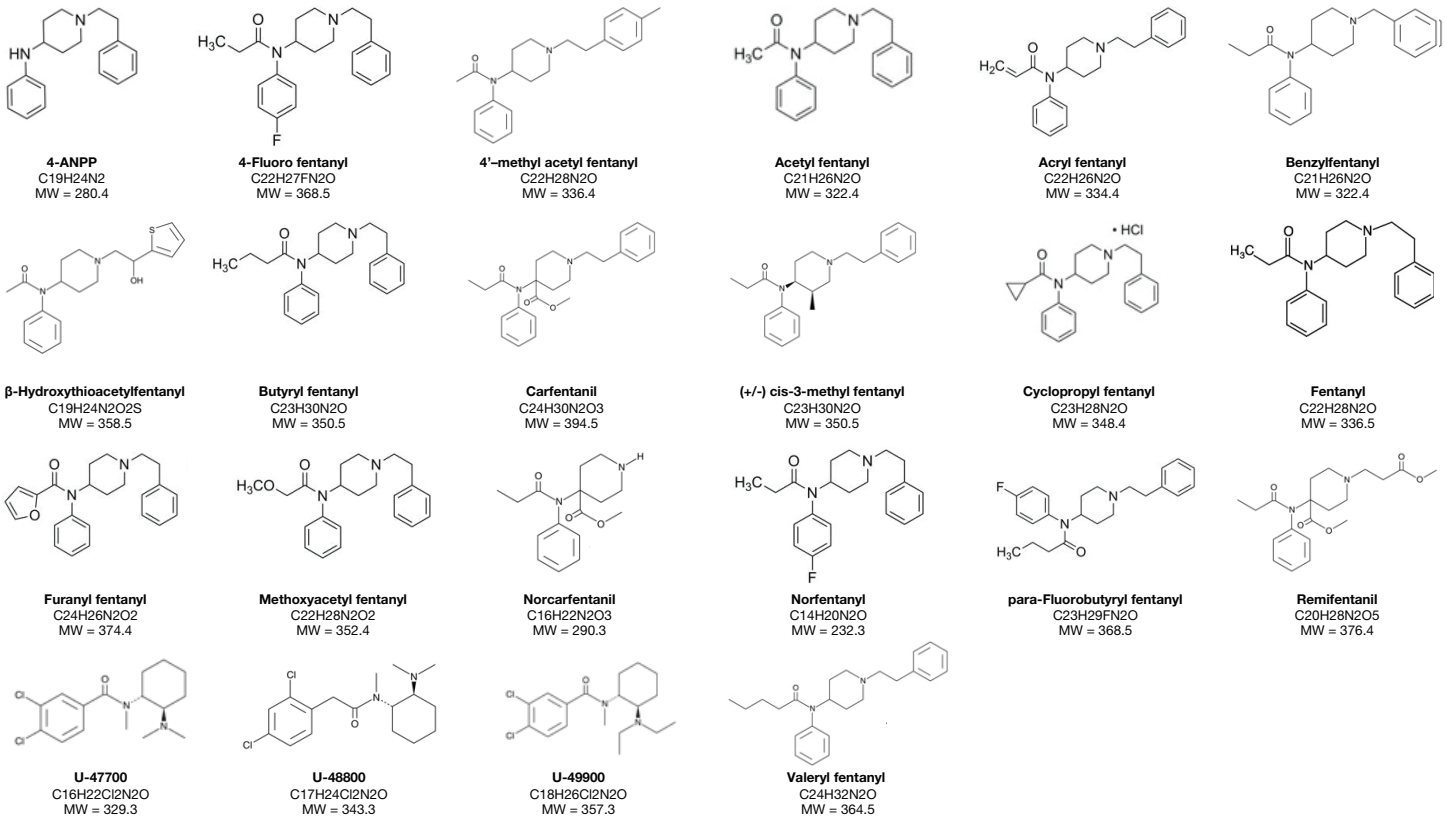

Figure 1. Fentanyl analogs: 22 synthetic opioids with internal standards quantitated

Calibration standards and control samples

All non-labeled standards were combined into a stock solution and diluted in methanol to a concentration of 10 µg/mL. Isotopically labeled standards were combined and diluted in methanol to a concentration of 10 µg/mL as well. A concentrated calibration solution for the non-labeled standard was prepared in human urine by diluting the non-labeled standard mix with human urine to a final concentration of 100 ng/mL. A 13-point calibration curve ranging from 0.01 ng/mL to 100 ng/mL was prepared by serial dilution with human urine as the diluent. Quality control (QC) standards were prepared at a concentration of 1.0 ng/mL, 2.5 ng/mL, and 25 ng/mL in urine. A 25 ng/mL internal standard (IS) working solution was created by diluting the primary IS stock solution in water. The IS working solution was added later to the non-labeled standards prior to sample preparation by SLE for absolute quantitation.

Supported liquid extraction preparation

Each of the standards in the 13-point calibration curve and the QC standards was processed by supported liquid extraction (SLE) with the Biotage™ ISOLUTE™ SLE+400 plates prior to analysis. A 200 µL aliquot was taken from each of the calibration and QC standards and was combined with 25 µL of the IS working solution and 175 µL of 0.2 % v/v formic acid in water in a microcentrifuge tube and vortexed for 5 seconds. The diluted standard was then pipetted onto the SLE plate with a 2 mL deep-well collection plate on a positive pressure manifold. The sample was allowed to absorb onto the SLE plate material for five minutes, and then 900 µL of dichloromethane (DCM) was added, without pressure, for five minutes until a short burst of air was applied at 0.7 bar positive to the SLE plate for elution of the entire aliquot of DCM. Next, an additional aliquot of DCM was added, without pressure, for five minutes, and then a short burst of air was applied at 0.7 bar of pressure to the SLE plate. The collection plate was removed, and the solvent was evaporated to dryness under nitrogen gas at a temperature of 50 °C. The dried samples were then resuspended with 100 µL of water:methanol (90:10) (v:v). The 96 deep-well plate was sealed with foil and shaken at 1,000 rpm for five minutes. The samples were then transferred to autosampler vials with glass inserts for analysis.

Liquid chromatography

A chromatographic method of 7.5 minutes was used for the analysis of fentanyl and fentanyl analog compounds using a Thermo Scientific™ Vanquish™ MD HPLC system consisting of a high pressure mixing gradient binary pump, a column compartment, and an autosampler. The separation was performed on a Thermo Scientific™ Accucore™ Phenyl Hexyl column (2.1 mm x 100 mm, 2.6 µm) maintained at 40 °C.

Mobile phases consisted of 2 mM ammonium formate in water with 0.1% formic acid for mobile phase A and a mixture of two (2) mM ammonium formate in methanol: acetonitrile (50:50 v:v) with 0.1% formic acid for mobile phase B. Chromatographic separation of a 10 µL injected sample was achieved by gradient elution under the conditions described in Table 1.

Table 1. LC gradient

Time (min)	Flow rate (mL/min)	% A	% B
0	0.5	80	20
1	0.5	80	20
4.5	0.5	5	95
5.5	0.5	5	95
7.5	0.5	80	20

Mass spectrometry

Compounds were detected by a TSQ Altis MD mass spectrometer with a Thermo Scientific™ OptaMax™ NG ion source with a heated electrospray ionization probe. The mass spectrometer source and scan settings are listed in Table 2 and Table 3, respectively. Selected reaction monitoring (SRM) mode was used for quantitation with a resolution of 0.7 full width half max (FWHM) for both quadrupoles and a 0.7 second cycle time with three transitions, one for quant and two for confirmation fragment ions for each precursor.

Table 2. Source parameters

Source parameter	Value
Positive ion	3,500 V
Sheath gas	55 AU
Aux gas	10 AU
Sweep gas	1 AU
Ion transfer tube temp.	325 °C
Vaporizer temp.	350 °C
Source position	1.5, M

Table 3. MS scan parameters

Compound	Retention time (min)	Precursor (*m/z*)	Product (*m/z*)	Min dwell time (ms)	RF lens (V)	Collision energy (V)	Quan
4-ANPP	3.07	281.201	188.300	5.244	63	16	X
			77.000			55	
			105.100			27	
4-Fluoro fentanyl	3.34	355.218	188.173	5.244	81	24	X
			105.125			38	
			150.000			33	
4’ methyl acetyl fentanyl	3.23	337.2274	119.054	5.244	88	34	X
			202.048			24	
Acetyl fentanyl	2.88	323.212	188.292	6.789	68	21	X
			102.952			56	
			105.137			34	
Acryl fentanyl	3.19	335.2118	188.292	5.244	79	21	X
			103.000			53	
			105.125			37	
Benzyl fentanyl	3.07	323.218	174.173	5.244	79	22	X
			91.054			37	
			131.952			32	
β-hydroxythiofentanyl	2.83	359.179	110.952	7.071	83	38	X
			146.000			25	
			191.952			24	
Butyryl fentanyl	3.56	351.243	188.173	5.519	71	24	X
			103.000			55	
			281.268			20	

Table 3. MS scan parameters (continued)

Compound	Retention time (min)	Precursor (*m/z*)	Product (*m/z*)	Min dwell time (ms)	RF lens (V)	Collision energy (V)	Quan
Carfentanil	3.49	395.233	335.196	5.244	80	18	X
			113.000			33	
			363.220			13	
cis-3-Methyl fentanyl	3.48	351.243	202.173	5.244	88	24	X
			105.000			37	
			134.000			30	
Cyclopropyl fentanyl	3.43	337.227	188.292	5.244	79	24	X
			105.000			34	
Fentanyl	3.26	337.227	188.048	5.244	80	24	X
			105.196			34	
Furanyl fentanyl	3.36	375.207	188.173	5.244	86	23	X
			102.952			55	
			105.000			40	
Methoxyacetyl fentanyl	2.81	353.222	188.173	7.532	83	22	X
			103.000			55	
			105.125			38	
Norcarfentanil	2.13	291.170	231.208	29.852	58	14	X
			146.000			29	
			259.280			11	
Norfentanyl	1.54	233.165	84.125	70.056	59	19	X
			55.054			35	
			55.952			28	
			177.048			17	
para-Fluorobutyryl fentanyl	3.63	369.234	188.173	5.817	89	26	X
			105.000			40	
			299.095			22	
Remifentanil	2.59	377.207	317.095	11.124	64	17	X
			345.220			31	
			285.048			21	
U-47700	3.11	363.191	284.077	5.244	69	17	X
			172.982			33	
			203.905			27	
U-48800	3.33	329.118	298.125	5.244	67	18	X
			112.048			31	
			218.030			27	
U-49900	3.34	357.150	284.054	5.244	71	19	X
			172.982			35	
			203.905			27	
Valeryl fentanyl	3.85	365.259	188.083	15.809	90	24	X
			105.000			39	
			244.083			24	

Method evaluation and data analysis

Quantitative data for the 22 fentanyl analytes were processed in Thermo Scientific™ TraceFinder™ LDT 1.0 software to determine the limit of quantitation (LOQ) as shown in Figure 2. The LOQ for each analyte was determined as the lowest value in the calibration curve giving an average % bias between nominal and back calculated concentration within ± 20% and a % CV below 20% for three replicate injections of calibrators.

Results and discussion

All 22 fentanyl analogs eluted between 1.5 and four minutes as shown in Figure 3. Internal calibration was used for all 22 fentanyl compounds with each fentanyl standard having a corresponding stable-isotope-labeled internal standard. Details of calibration approach and LOQ for each analyte are reported in Table 4. The TSQ Altis MD demonstrated LOQs ≤ 0.25 ng/mL for all but two out of the 22 fentanyl compounds, and all had an R^2 above 0.99. Representative chromatograms for the quantitative and confirming ions for the lowest calibrator for benzyl fentanyl (0.1 ng/mL) and valeryl fentanyl (5 ng/mL) are shown in Figure 4 with calibration curves shown in Figure 5.

Figure 2. Data analysis performed with TraceFinder LDT 1.0 software for 22 fentanyl analogs. Compound list table is shown (A), followed by sample results (B), quan peak (C), confirming ion peak (D), and calibration curve (E).

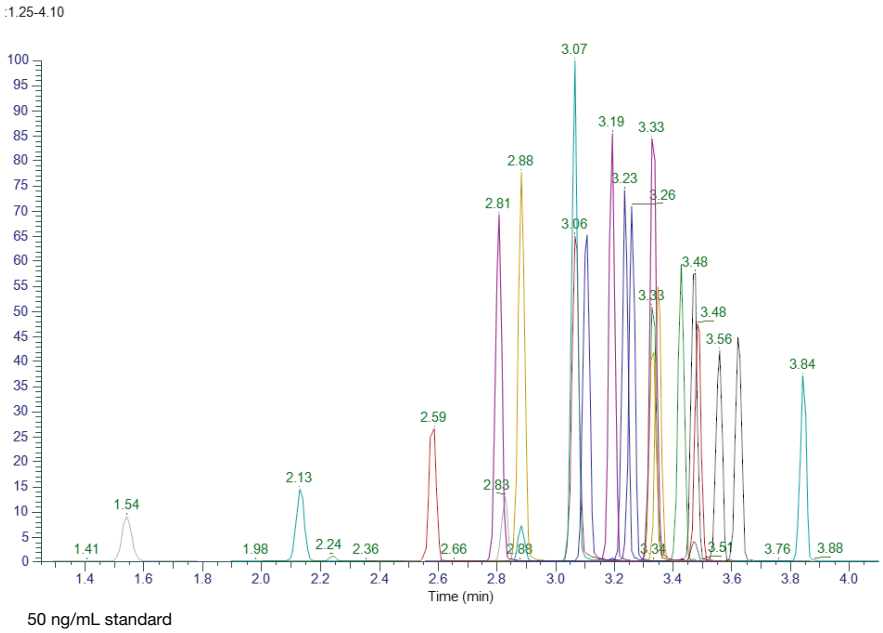

Figure 3. Chromatographic separation of 22 fentanyl analogs. The first four minutes are shown of the total runtime.

Table 4. Limit of quantitation. The compound of interest and corresponding internal standard is listed with results for lower limit of quantitation and linearity range. All compounds were detected linearly with $1/X^2$ weighting to a maximum concentration of 100 ng/mL, unless noted, with the origin ignored.

Compound	Internal standard	LLOQ (ng/mL)	Linearity range (ng/mL)	Type	Weighting	Origin
4-ANPP	4-ANPP-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
4-Fluoro fentanyl	4-Fluoro fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
4' methyl acetyl fentanyl	4'methyl acetyl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Acetyl fentanyl	Acetyl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	1/X	Ignore
Acryl fentanyl	Acryl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Benzyl fentanyl	Benzyl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
β-Hydroxythiofentanyl	β-Hydroxythiofentanyl-$^{13}C_6$	0.25	0.25–100	Linear	$1/X^2$	Ignore
Butyryl fentanyl	Butyryl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Carfentanil	Carfentanil-$^{13}C_6$	0.25	0.25–100	Linear	$1/X^2$	Ignore
cis-3-Methyl fentanyl	3-Methyl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Cyclopropyl fentanyl	Cyclopropyl fentanyl-$^{13}C_6$	0.05	0.05–50	Linear	$1/X^2$	Ignore
Fentanyl	Fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Furanyl fentanyl	Furanyl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Methoxyacetyl fentanyl	Methyoxyacetyl fentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Norcarfentanil	Norcarfentanil-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
Norfentanyl	Norfentanyl-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
para-Fluorobutyryl fentanyl	*para*-Fluorobutyryl fentanyl-$^{13}C_6$	0.5	0.5–50	Linear	$1/X^2$	Ignore
Remifentanil	Remifentanil-$^{13}C_6$	0.1	0.1–100	Linear	$1/X^2$	Ignore
U-47700	U-47700-$^{13}C_2$,$^{15}N_2$	0.25	0.25–100	Linear	$1/X^2$	Ignore
U-48800	U-48800-$^{13}C_3$,$^{15}N_2$	0.25	0.25–100	Linear	$1/X^2$	Ignore
U-49900	U-49900-$^{13}C_5$	0.05	0.05–100	Linear	$1/X^2$	Ignore
Valeryl fentanyl	Valeryl fentanyl-$^{13}C_6$	5	5–100	Linear	1/X	Ignore

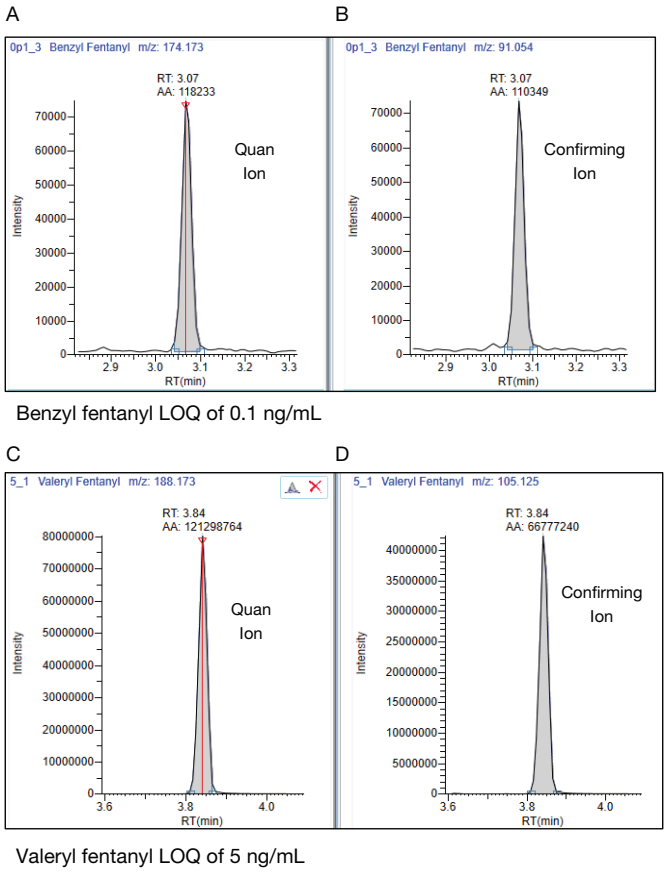

Figure 4. Quantitative and confirming ions for benzyl fentanyl (A, B) and valeryl fentanyl (C, D) at the limit of quantitation

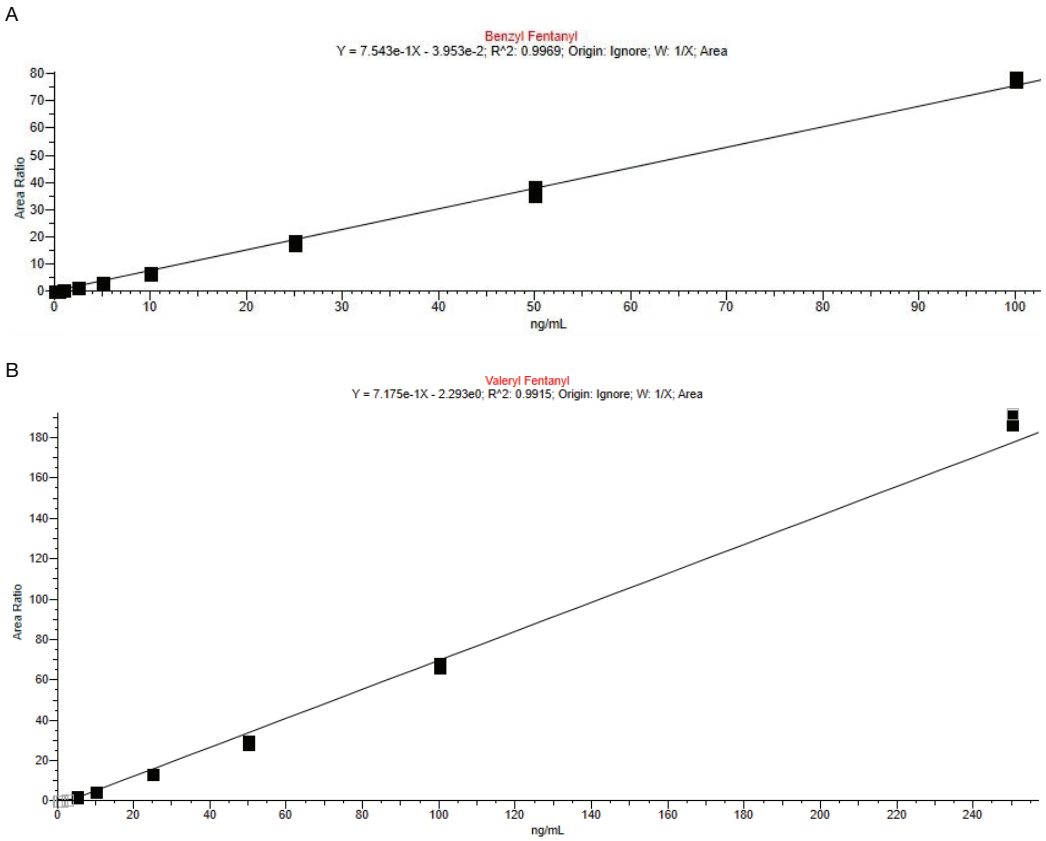

Figure 5. Calibration curves for benzyl fentanyl (A) and valeryl fentanyl (B) at the limit of quantitation

Conclusions

A sensitive and reproducible method for the quantitative measurement of 22 fentanyl compounds in urine was developed on the Vanquish MD HPLC system and TSQ Altis MD mass spectrometer. Sample preparation involved sample cleanup by SLE followed by a 7.5-minute UHPLC gradient separation and detection by a triple quadrupole mass spectrometer. Detection limits were ≤ 0.25 ng/mL for 20 of the 22 compounds tested.

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