Flunitrazepam Excretion Patterns using the Abuscreen OnTrak and OnLine Immunoassays: Comparison with GC–MS

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Abstract

A study was conducted to compare the performance of the OnLine and OnTrak immunoassays for benzodiazepines with gas chromatographic–mass spectrometric (GC–MS) analysis in detecting flunitrazepam (FNP) and its metabolites in human urine. Urine was collected over a 72-h period from six individuals (four male and two female) who had taken a single oral dose of either 1 or 4 mg of FNP. The OnTrak assay was run at a 100-ng/mL cutoff of nordiazepam (NDP), and the OnLine assay was run with a standard curve from zero to 200 ng/mL of NDP with and without β-glucuronidase treatment. Each sample was analyzed by GC–MS using FNP, 7-amino-FNP, 3-hydroxy-FNP, desmethyl-FNP, 7-amino-3-hydroxy-FNP, and desmethyl-3-hydroxy-FNP as standards with β-glucuronidase treatment. The specimens from the 1-mg dose did not yield a positive result by immunoassay over the 72-h collection period. Specimens from the 4-mg dose did yield positive results in both immunoassays. The time of the first positive result ranged from 4 to 12 h, and the time to the last positive result ranged from 18 to 60 h. Treatment of the samples with β-glucuronidase increased the OnLine values between 20 and 60%, but it did not appreciably increase the detection time. GC–MS analysis showed no detectable levels of FNP, 3-hydroxy-FNP, desmethyl-FNP, 7-amino-3-hydroxy-FNP, and desmethyl-3-hydroxy-FNP. However, all samples collected past time zero showed detectable levels of 7-amino-FNP (> 2 ng/mL) with peak concentrations at 12–36 h. The peak levels of 7-amino-FNP by GC–MS paralleled the peak levels of the immunoassay response. The amount of 7-amino-FNP metabolite quantitated by GC–MS, however, accounted for only 15–20% of the total immunoassay crossreactive FNP metabolites.

Introduction

Flunitrazepam (FNP) is a prescription sleeping aid that is one of a widely used class of prescription medications known as benzodiazepines (1). The drug is sold under the trade name Rohypnol and is distributed in 64 countries throughout the world. It is one of the more commonly used benzodiazepines in certain countries, and its abuse is evident in the intravenous drug population (2). The drug is not sold in the United States, but there have been increasing reports that the drug is being illegally diverted into the country and illicitly sold (3).

Chromatographic methods such as gas chromatography and liquid chromatography were developed to determine FNP and its metabolites in various body fluids (1,4). However, the detection of flunitrazepam in urine has been of concern to toxicologists for several years (5–7). Although commercially available assays show some crossreactivity to FNP, very little of the parent drug appears in urine (8). It is extensively metabolized by reduction of the nitro group followed by acetylation, hydroxylation of the 3-position followed by glucuronidation, and *N*-demethylation (Figure 1). In addition to the complicated metabolism of FNP, the lower therapeutic doses of this compound compared with the older generation of benzodiazepines make its detection in urine difficult.

Although a controlled study was conducted to determine the excretion pattern of FNP by immunoassays (7), there are no available studies that compare the excretion patterns of FNP with immunoassays followed by gas chromatographic–mass spectrometric (GC–MS) analysis. In this study, we compare the excretion profiles of six individuals who have taken either a 1- or 4-mg dose of FNP by the OnTrak and OnLine immunoassays (Roche Diagnostic Systems, Somerville, NJ) followed by GC–MS.

Experimental

Materials

FNP, 7-amino-FNP, 3-hydroxy-FNP, desmethyl-FNP, 7-amino-3-hydroxy-FNP, and desmethyl-3-hydroxy-FNP were all supplied by Roche Laboratories (Nutley, NJ). β-Glucuronidase (β-glucuronide glucuronosohydrolase, EC 3.2.1.31) was purchased from Boehringer Mannheim (Indianapolis, IN). The Cobas Integra was provided by Roche Diagnostic Systems.

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Biological samples

This study was approved by the Ethics Committee of the Faculty of Medicine from the University of Bern. All subjects that participated in the study were volunteers and supplied informed consent. Four healthy male subjects (30–47 years of age, 59–87 kg in weight) and two healthy female subjects (25–40 years of age, 55–72 kg in weight) participated in the study. A single oral dose of 1 or 4 mg of FNP was ingested in the evening by each subject. Urine specimens were collected, and the volume was measured at the time of FNP administration (time 0 h) and at 2, 4, 8, 12, 18, 24, 30, 36, 48, 60, and 72 h after drug ingestion. Specimens were collected in polypropylene bottles without additives and immediately refrigerated. All specimens from the study were frozen at –24°C within 12 h of collection.

Immunological assays

Benzodiazepine kits for the OnTrak and OnLine assays were obtained from Roche Diagnostic Systems. The OnTrak assay is a single test qualitative assay with a 100-ng/mL cutoff using nordiazepam as a standard. The OnLine assay was run on the Cobas Integra in the quantitative mode using a four-point linear interpolation standard curve based on nordiazepam (0, 50, 100, and 200 ng/mL) calibrators. The OnLine assay was run in two different modes according to manufacturer's instructions. One mode used the standard assay without pretreatment of the urine sample. The second mode used a β-glucuronidase pretreatment step that is incorporated directly on the instrument. The β-glucuronidase (0.4 units) is incubated with the sample, sample diluent, and antibody for 2.6 min before the instrument adds the start reagent. The clinical limit of detection for both modes of the OnLine assays was determined to be 26 ng/mL. The intra-assay coefficient of variation (CV) for both assays at the 50 ng/mL level averaged 7.1% (n = 20), and the interassay CV at the 50 ng/mL level averaged 7.6% (n = 200).

GC–MS analysis

Urine samples were hydrolyzed with β-glucuronidase and extracted with chloroform–isopropanol (9:1). For the best chromatographic separation and the least interference, trimethylsilyl (TMS) derivatization was chosen for 7-amino-FNP, 3-hydroxy-FNP, and desmethyl-FNP (group I). Tertiarybutyldimethylsilyl trifluoroacetamide (TBDMS) derivatization was chosen for 7-amino-3-hydroxy-FNP and desmethyl-3-hydroxy-FNP (group II). Even though flunitrazepam itself was not derivatized by either method, it was analyzed with group I compounds. Oxazepam-d_5 was used as the internal standard.

The identification and quantitation of the analytes were performed using selected ion monitoring (SIM). The ions selected for this purpose were chosen from full scan mass spectral analysis of 5 µg/mL standard of each analyte. Two ions were chosen to monitor the presence or absence of each analyte. A linear calibration curve of 10, 25, 50, 150, and 300 ng/mL was used for 7-amino-FNP and 7-amino-3-hydroxy-FNP; a calibration curve of 50, 150, and 300 ng/mL was used for the other analytes. A negative control was included along with an unextracted standard to calculate the extraction recovery. Specimens and controls had to have retention times and ion ratios within ± 2% and 20%, respectively, of the standards.

The following procedure was used: internal standard (30 µL) containing 40 µg/mL of oxazepam-d_5 was added to 4 mL urine in a 15-mL centrifuge tube. Acetate buffer (1.0 mL, 1.1M, pH 5.2) was added to each tube. One hundred microliters of glusulase (DuPont, containing 10,000 units β-glucuronidase/mL and approximately 9000 units/mL sulfatase) was added to each tube. The tubes were capped loosely and vortex mixed briefly, and then placed in a 37°C oven for 4 h. After hydrolysis, the tubes were removed from the oven and allowed to cool to room temperature. One milliliter of phosphate buffer (40%, pH 9.0) was added to each tube. Eight milliliters of chloroform–isopropanol (9:1) was added to each tube. The tubes were then shaken for 2 min. If necessary, the tubes were centrifuged to break the emulsion. The aqueous layer was discarded, and the organic layer was shaken with 1 mL distilled water. The aqueous layer was again discarded. The organic layer was poured into 13 × 100-mm tubes and evaporated under nitrogen at 50°C to a volume of approximately 1 mL. This volume was transferred to GC vials, and the solvent was evaporated under nitrogen.

For group I analytes, 100 µL BSTFA (containing 1% TMCS) was added, and the vials

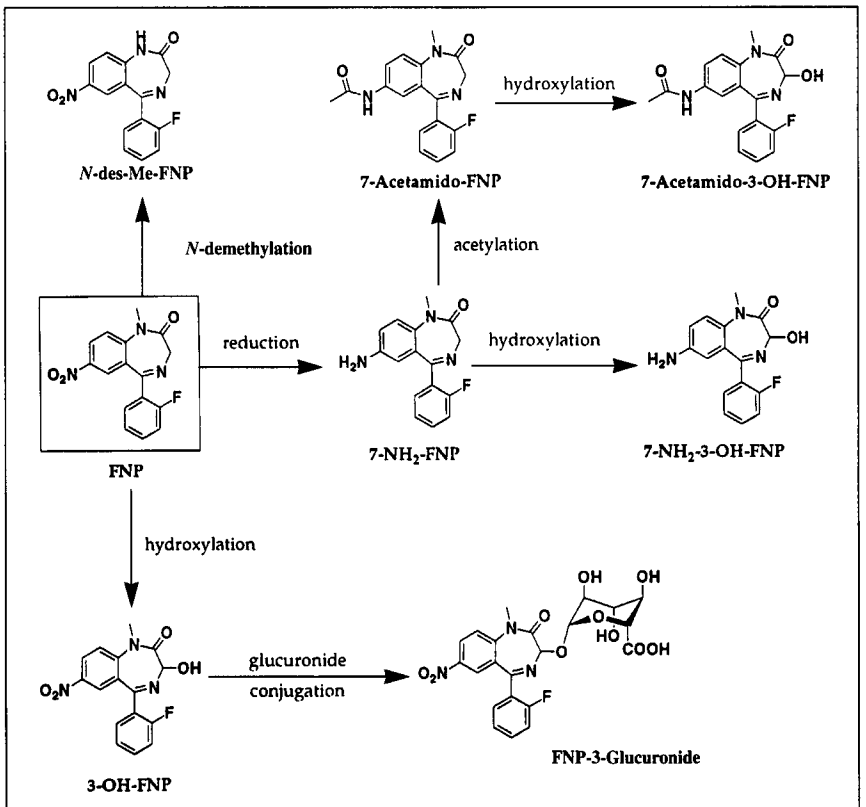

Figure 1. Flunitrazepam metabolism in humans.

were capped and placed in a 70°C oven for 30 min. The vials were removed from the oven and cooled to room temperature. The samples were then transferred into a GC insert and capped. The samples (2 µL) were injected onto the GC–MS. For group II analytes, 75 µL of MTBSTFA (*N*-tert-butyldimethylsilyl)-*N*-methyl trifluoroacetamide with 1% TBDMSC1 was added instead of BSTFA. The derivatization time and temperature were the same as those for BSTFA. A GC-vial insert was also used. Two microliters of samples were also injected on GC–MS.

A Hewlett Packard 5890 GC with an HP 5970 mass selective detector and a Chemstation data system were used in electron ionization mode (70 eV) with SIM. To analyze group I analytes, a 10-m × 0.18-mm (0.4-µm film thickness) DB-1 column (J&W Scientific, Folsom, CA) was used (Table I). The temperature was programmed at 180°C (held for 0.5 min) to 280° at 30°C/min (held for 6 min) and to 300°C at 30°C/min (held for 1.5 min). The samples (2 µL) were injected in the splitless mode with the valve closed for 0.2 min. The splitless injector temperature was 250°C, and the detector temperature was 280°C. The helium carrier flow was 43 cm/s.

To analyze group II compounds (Table I), a 25-m × 0.2-mm (0.33-µm film thickness) DB-5 MS column (J&W Scientific) was operated under similar conditions, but the temperature was programmed as follows: 220°C (held for 0.2 min) to 280°C at 30°C/min (held for 12.5 min).

Results

The immunoassay data shows that with the 1-mg dose, the urine concentration was below the clinical limit of detection in all three individuals with the unmodified OnLine assay over the 72-h period. Using the β-glucuronidase pretreatment, several samples showed low concentrations of crossreactive benzodiazepines with the OnLine assay. The values ranged between 34 and 43 ng/mL and were found between 12 and 30 h (Table II). The OnTrak assay with a 100-ng/mL cutoff gave negative results with all the 1-mg dose subjects throughout the 72-h collection period.

With the 1-mg dose, the GC–MS analysis was able to detect the presence of the 7-amino-FNP metabolite at concentrations ranging from 2 to 58 ng/mL over the entire 72-h period with the highest concentrations appearing between 12 and 60 h (Table II). The parent drug (FNP) and the four other metabolites were not detected by GC–MS.

The immunoassay results with the 4-mg dose showed levels of crossreactive benzodiazepines substantially higher than those of the 1-mg dose. The unmodified OnLine assay registered levels ranging from 30 to 172 ng/mL between 8 and 60 h. The modified assay using β-glucuronidase gave values that were 20 to 80% higher than the unmodified assay (Table III). The OnTrak assay gave positive results for these samples between 8 and 60 h. Figure 2 shows the excretion profiles of the three 4-mg dose subjects with and without β-glucuronidase treatment. Normalizing the values for creatinine concentrations did not change the excretion profile considerably.

The GC–MS values for the 4-mg dose subjects were also substantially higher than for the 1-mg dose subjects. Concentrations of 7-amino-FNP ranged from 2 to 118 ng/mL over the 72-h period with maximum levels appearing between 8 and 60 h (Table III). The excretion profiles for all three 4-mg dose subjects using the 7-amino-FNP GC–MS concentrations are shown

Table I. Retention Times and Ions Monitored for Group I and Group II Analytes

Compounds	Retention time (min)	Ions
Group I (TMS derivatives) on DB-1 column		
Oxazepam-d_5*	4.196	420, 434 (QI)†
Desmethyl-FNP	4.575	352, 371 (QI)
Flunitrazepam (FNP)	5.103	286, 312 (QI)
7-Amino-FNP	5.548	327, 355 (QI)
3-Hydroxy-FNP	5.803	386, 372 (QI)
Group II (TBDMS derivatives) on DB-5 column		
Oxazepam-d_5*	7.708	519, 462 (QI)†
Desmethyl-3-Hydroxy-FNP	11.661	487, 486 (QI)
7-Amino-3-Hydroxy-FNP	12.809	282, 356 (QI)

* Internal standard.
† Quantitation ion.

Table II. Urinary Excretion of Flunitrazepam after a 1-mg Dose in Three Individuals

Patient sample	Time after ingestion (h)	GC–MS 7-AFNP	OnLine-Cobas Integra 0.0 U Enz. conc.*	OnLine-Cobas Integra 0.4 U Enz. conc.*	OnTrak 100-ng/mL cutoff
A1	0	0	<0	<0	–
A2	2	3	<0	<0	–
A3	12	58	19	43	–/+
A4	18	12	<0	<0	–
A5	24	24	2	7	–
A6	30	23	25	34	–
A7	36	19	4	9	–
A8	48	7	12	16	–
A9	60	8	<0	<0	–
A10	72	2	<0	<0	–
B11	0	0	<0	<0	–
B12	8	8	<0	<0	–
B13	12	7	<0	<0	–
B14	18	23	7	13	–
B15	30	27	31	42	–
B16	36	11	17	22	–
B17	48	14	<0	<0	–
B18	60	14	4	6	–
B19	72	9	<0	<0	–
C20	0	0	<0	<0	–
C21	18	9	<0	<0	–
C22	24	7	<0	<0	–
C23	30	6	<0	<0	–
C24	36	6	<0	<0	–
C25	48	13	<0	<0	–
C26	60	24	2	8	–
C27	72	5	<0	<0	–

* ng/mL.

in Figure 3. FNP, desmethyl-FNP, desmethyl-3-hydroxy-FNP, and 7-amino-3-hydroxy-FNP were not detected in any of the samples. The 3-hydroxy-FNP metabolite was detected in one individual between 8 and 24 h at concentrations between 60 and 93 ng/mL. It was also detected in the 12-h void of another individual at a concentration of 68 ng/mL.

Conclusion

The immunoassay data show that 1-mg doses of FNP were difficult to detect in urine at any level. The use of β-glucuronidase in the assay increased the sensitivity, but the detection window was very narrow, and the levels remained below 50 ng/mL. The immunoassay levels in urine samples from individuals that have taken a 4-mg dose were substantially higher and could readily be detected at concentrations above 50 ng/mL. The use of β-glucuronidase in the immunoassay converted the glucuronidated benzodiazepine metabolite, with low crossreactivity, into an unconjugated molecule that has higher crossreactivity. The treatment increased the immunoassay values by 20 to 80% (Tables II and III). The use of β-glucuronidase treatment to increase benzodiazepine immunoassay sensitivity is not a new method and has been previously reported (5,7,9,10).

The OnTrak assay, which has a cutoff of 100 ng/mL, identified all the OnLine samples that had values above 30 ng/mL as positive. The reason for this increased sensitivity could be explained by the difference in the OnTrak and OnLine antibody crossreactivities. The OnTrak antibody had a higher crossreactivity to FNP, 7-amino-FNP, 7-amino-3-hydroxy-FNP, and 7-amino-1-desmethyl-FNP than did the OnLine antibody (Table IV). This difference was sufficient to increase the assay sensitivity without the need for β-glucuronidase treatment.

The GC–MS results indicate that the 7-amino-FNP metabolite

Table III. Urinary Excretion of Flunitrazepam after a 4-mg Dose in Three Individuals

Patient sample	Time after ingestion (h)	GC–MS 7-AFNP	OnLine-Cobas Integra 0.0 U Enz. conc.*	OnLine-Cobas Integra 0.4 U Enz. conc.*	OnTrak 100-ng/mL cutoff
D40	0	0	<0	<0	–
D41	8	71	30	74	+
D42	12	42	86	**154**†	+
D43	18	3	45	79	–
D44	24	68	**107**	**154**	+
D45	30	67	97	**124**	+
D46	36	13	1	9	–
D47	48	34	0	9	–
D48	60	2	<0	<0	–
D49	72	2	<0	<0	–
E50	0	0	<0	<0	–
E51	2	16	<0	<0	–
E52	4	44	21	41	+
E53	8	80	**108**	**169**	+
E54	12	118	**178**	**183**	+
E55	18	104	**172**	**>200**	+
E56	24	63	**111**	**136**	+
E57	30	67	**124**	**165**	+
E58	36	81	**132**	**170**	+
E59	48	65	48	74	+
E60	60	59	59	93	+
E61	72	23	4	10	–
F62	0	0	1	1	–
F63	12	110	**124**	**167**	+
F64	18	46	9	27	–
F65	24	55	20	40	–
F66	30	35	39	66	+
F67	36	84	**139**	**182**	+
F68	48	25	0	7	–
F69	60	28	29	39	+
F70	72	18	8	7	–

* ng/mL.
† Bold numbers indicate results that are greater than or equal to the normal urine immunoassay cutoff of 100 ng/mL.

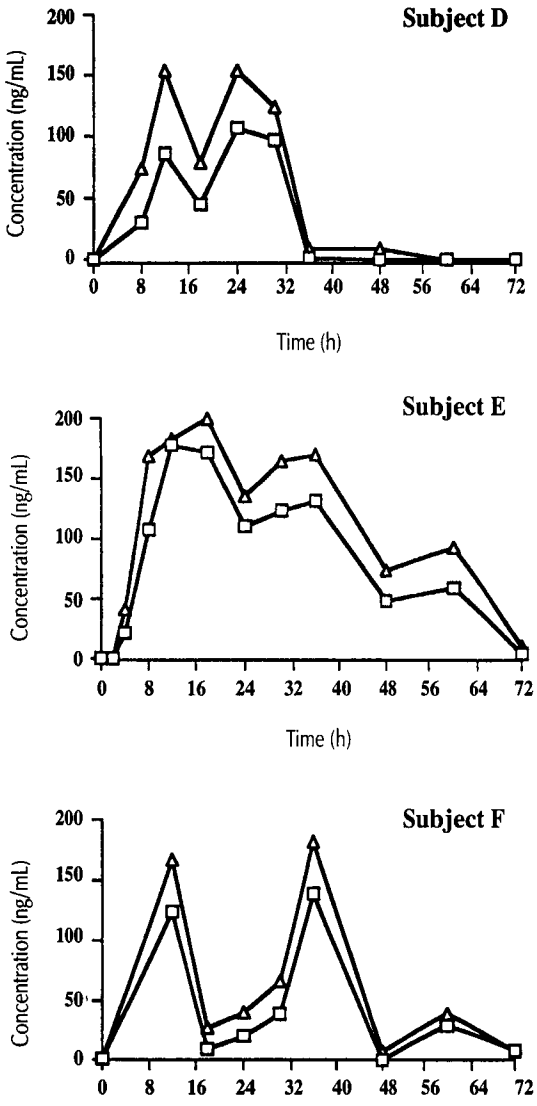

Figure 2. Urinary excretion profile (nordiazepam equivalents) of subjects D, E, and F after a 4-mg dose using OnLine with (△) and without (□) β-glucuronidase treatment.

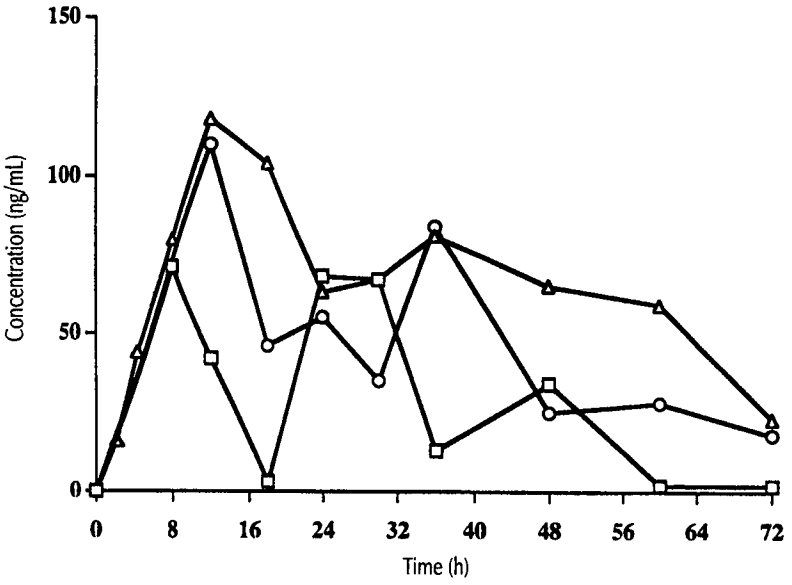

Figure 3. Urinary excretion profile of 7-amino-FNP of individuals D (□), E (△), and F (○) after a 4-mg dose using GC–MS with β-glucuronidase hydrolysis.

Table IV. Cross-Reactivity of FNP and its Metabolites in the OnLine and OnTrak Immunoassays Relative to 100 ng/mL of Nordiazepam

Compounds	OnLine	OnTrak
Flunitrazepam	55%	80%
7-Amino-flunitrazepam	30%	80%
3-Hydroxy-flunitrazepam	26%	27%
Desmethyl-flunitrazepam	59%	59%
7-Amino-3-hydroxy-flunitrazepam	11%	20%
7-Amino-1-desmethyl-flunitrazepam	10%	50%

is the most abundant metabolite out of the five metabolites studied. Out of the other four metabolites, only the 3-hydroxy-FNP metabolite was observed and then only in a few samples. The values of the 7-amino-FNP metabolite paralleled those of the immunoassay values. Using GC–MS with a low limit of detection (2 ng/mL) allowed for confirmation of 7-amino-FNP up to 72 h with doses as low as 1 mg.

The OnLine immunoassay values were substantially higher than the GC–MS values for 7-amino-FNP. Because the OnLine antibody had 30% crossreactivity to the 7-amino-FNP metabolite, it indicated that there was a considerable amount of metabolite that was not being identified. The 7-amino-FNP metabolite accounts for only 15–20% of the total crossreactive benzodiazepine metabolites detected by the immunoassay. An alternative GC–MS procedure in which the metabolites were hydrolyzed to the corresponding benzophenone derivatives was developed (11). This procedure gave values that better accounted for the total immuno-crossreactive benzodiazepines found in the clinical samples.

The use of β-glucuronidase treatment in the detection of flunitrazepam use was best seen in cases where lower doses of the drug are used. This could be seen in cases such as date rape in which a limited number of tablets were added to a victim's drink. The immunoassay values of these samples were generally low, and enzyme treatment increased the window of detection (unpublished data). In driving-under-the-influence (DUI) cases, the levels of FNP metabolites were generally far higher (12), and the use of β-glucuronidase treatment was less critical. In summary, the use of β-glucuronidase treatment in immunoassays increased the window of detection for all uses of the drug and especially aided in the detection of FNP use when low amounts of the drug were taken.

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