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Fruitful Application of Steroid Methodology for Drug of abuse Testing: Comprehensive Benzodiazepine Screening and Confirmation

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Introduction

Benzodiazepines are the most popular pharmacological agents in society, both as addictive drugs and as useful therapeutic substances. Being a source of abuse, they are classified as controlled substances in the United States. Benzodiazepines may seriously affect human performance in safety sensitive jobs due to their sedating effect, consequently, they are a part of routine employment drug testing. In sports, they may be misused in such disciplines as shooting and modern pentathlon, where they are considered doping. Clinical applications of benzodiazepines include sedation, anesthesia, muscle relaxation, treatment of anxiety, depression and insomnia.

The frequently used benzodiazepines worldwide are shown in Fig. 1. Based upon their structure and metabolism benzodiazepines fall into several groups:

- the broad category sharing oxazepam as common urinary metabolite;
- 7-nitro-benzodiazepines, nitrazepam, clonazepam, and flunitrazepam, each converting into specific metabolites, primarily 7-amino and 7-acetamido derivatives;
- annelated benzodiazepines with imidazole or triazole cycles attached at the 1,2 position, which metabolize by alpha-hydroxylation;
- the miscellaneous, bromazepam, clobazam, flurazepam, lorazepam.

Some benzodiazepines undergo conversion into appreciable amounts of benzophenones (nitrazepam, bromazepam). Most urinary metabolites are conjugated. Metabolism is described in numerous original papers and summarized in a monograph by Baselt and Cravey (1).

The drug testing industry traditionally relies on immunoassays for efficient benzodiazepine urine screening (2-10), because it is an efficient means of performing high volume testing at low cost. The assays are designed primarily for oxazepam related drugs. Some benzodiazepines show poor crossreactivity and remain undetected. The variety of benzodiazepine drugs available, each with multiple metabolites, makes it difficult to develop a comprehensive immunoassay equally suitable for all of them (11). The wide range of drug potencies and the consequent range of metabolic concentrations in urine further contribute to this problem. The relatively high cutoff limits of these assays frequently provide an unreasonable tolerance when applied to the positive detection of highly potent benzodiazepines.

Alternative procedures are available for selected drugs based on GC (12-24) or HPLC(25-29) methodology. Our approach has been to apply a modified version of the GC/MS screening technique, familiar as procedure IV for anabolic steroids, to achieve a truly comprehensive benzodiazepine screen.

Materials and methods

Positive excretion urines for each of the drugs listed in Fig. 1 and Table 1 were obtained from different sources including patients known to be taking benzodiazepines, and from volunteers.

Commercially available standards of benzodiazepines, their metabolites and deuterated substances were purchased from Sigma Co., Radian Corp. , and Alltech Assoc., Inc. B-Glucuronidase enzyme type H-3 (*Helix Pomatia*) was supplied by Sigma Co. N,O-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorsilane (BSTFA + 1% TMCS) was from Campbell Supply Co.

Enzyme/ internal standards/ acetate buffer - stock solution was prepared by mixing:

25 mL of β-glucuronidase
D5 α-hydroxyalprazolam ISTD 2 mL of 100 μg/mL in methanol
D5 temazepam ISTD 4 mL of 100 μg/mL
D5 nordiazepam ISTD 400 μL of 1 mg/mL
D5 oxazepam ISTD 400 μL of 1 mg/mL
468 mL of 1M acetate buffer, pH 5.2

Calibration standard: negative urine spiked with:

200 ng/mL of desalkylflurazepam, nordiazepam, oxazepam, lorazepam, 7-aminonitrazepam, 4'-hydroxynordiazepam, temazepam
300 ng/mL of 7-aminoclonazepam
500 ng/mL of 2-hydroxyethylflurazepam
100 ng/mL of 7-aminoflunitrazepam, α-hydroxyalprazolam, α-hydroxytriazolam

Procedure:

- Check urine pH, adjust to the normal range if necessary.
- Aliquot 2 mL urine, add 0.5 mL of combined enzyme/ISTD/buffer solution, incubate 3 hours at 52°C.
- Add 0.5 mL of 1 M sodium bicarbonate solution, vortex and centrifuge.
- Condition C_{18} columns by flushing once with 2 mL of methanol and once with 2 mL of water.
- Add urine samples and allow them to drain through.
- Rinse columns with 2 mL of 15% acetonitrile/water, apply vacuum to dry columns.
- Elute with 2 mL of methanol, evaporate to dryness.
- Add 100 μL of BSTFA + 1% TMCS. Incubate at 70°C for 20 minutes.
- Inject 1 μL into GC/MS.

GC/MS Parameters:

Hewlett-Packard 5890/5970 GC/MS with 7673 autosampler and unix/target software.
HP II fused silica column 12.5m x 0.2mm i.d., 0.33 μ 5% phenyl methylsilicon film thickness.

Instrument conditions:

Injection port:	270°C
Transfer line:	290°C
Oven:	180°C (0.5 min)
ramp to	320°C at 12 °C/min
hold at	320°C for 0.33 min

Results and Discussion

Table 1 summarizes data on studied benzodiazepines, their urinary metabolites, retention times, molecular weights and major identification ions.

Table 1.

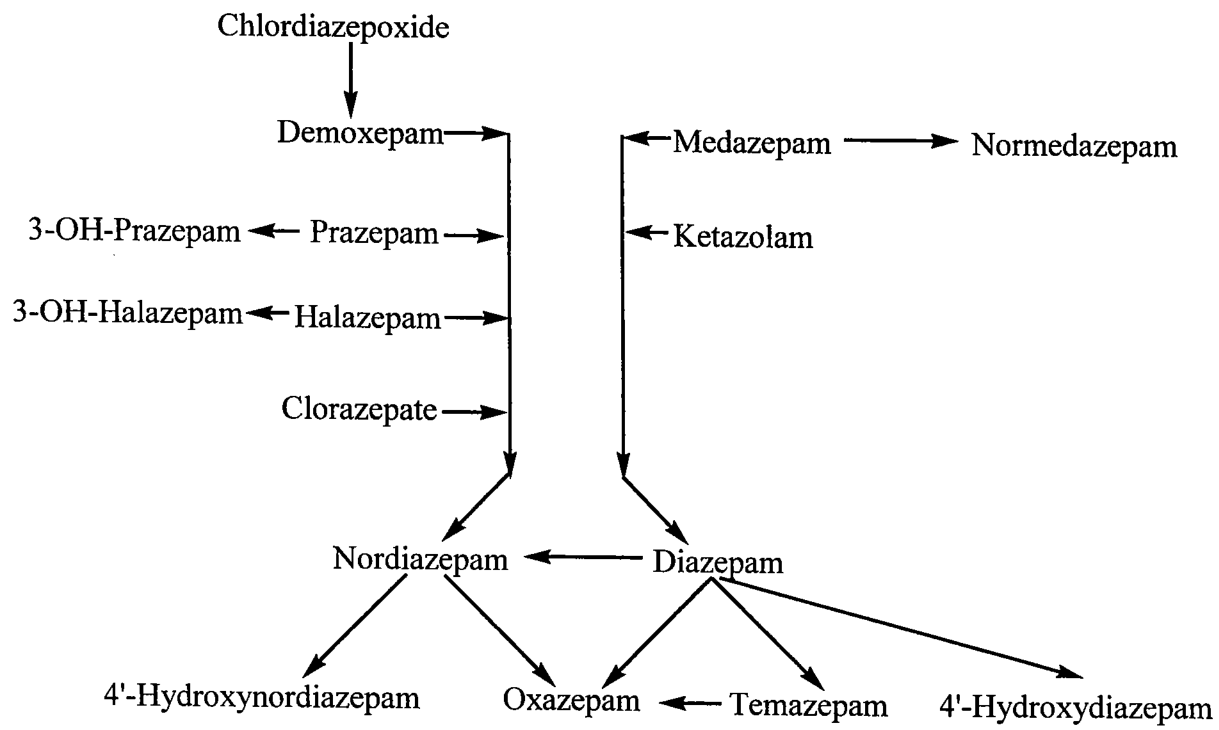
Benzodiazepines, their urinary metabolites, and GC/EI-MS data for the N- and O-TMS derivatives

Parent Drug *(Brand Name)*	**Major Urinary Metabolite(s)**	**R.T. min.**	**M^+ m/z**	**Essential ions and their relative abundances %**
Alprazolam *(Xanax)*	Hydroxyalprazolam	10.90	396	381(100), 396(40), 383(41)
Bromazepam *(Lexotan)*	1-OH-2-NH_2-5-Br-Benzophenone	7.22	436	423(100), 436 (8), 438 (8)
	3-Hydroxybromazepam	7.65	475/477	388(100), 475 (47), 477 (49)
Chlordiazepoxide *(Librium)*	Nordiazepam	5.90	342	342(64), 343 (54), 327 (21)
	4'-Hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
	Demoxepam			
Clobazam *(Frizium)*	4'-Hydroxyclobazam	9.97	388	388(100), 390 (67), 345 (70)
	4'-Hydroxynorclobazam	8.72	446	446(100), 448 (40), 431 (32)
Clonazepam *(Klonopin)*	7-Aminoclonazepam	8.46	429	429(100), 431 (50), 394 (94)
	7-Acetamidoclonazepam	8.71	471	471(100), 456 (70), 436 (90)
Clorazepate *(Tranxene)*	Nordiazepam	5.90	342	342 (64), 343 (54), 327 (21)
	4'-Hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
Delorazepam *(En)*	Lorazepam	7.36	464	429(100), 430 (36), 431 (45)
	4'-Hydroxydelorazepam	8.48	464	449(100), 541 (85), 464 (85)
Diazepam *(Valium)*	Nordiazepam	5.90	342	342 (64), 343 (54), 327 (21)
	4'-Hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
	Temazepam	8.16	372	372 (28), 373 (10), 374 (12)
Halazepam *(Paxipam)*	Nordiazepam	5.90	342	342 (64), 343 (54), 327 (21)
	4'-Hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)

	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
	3-Hydroxyhalazepam			
Flunitrazepam *(Rohypnol)*	7-Aminoflunitrazepam	8.74	355	354 (31), 327 (40), 326 (38)
Flurazepam *(Dalmane)*	Desethylflurazepam	5.79	359	359(100), 360 (98), 361 (70)
	2-Hydroxyethylflurazepam	8.54	404	288(100), 389 (36), 391 (16)
Lorazepam *(Ativan)*	Unchanged	7.36	464	429(100), 430 (36), 431 (45)
Lormetazepam *(Noctamid)*	Unchanged	8.70	406	377(100), 379 (72), 391 (19)
	Lorazepam	7.36	464	429(100), 430 (36), 431 (45)
Medazepam *(Nobrium)*	Nordiazepam	5.90	342	342 (64), 343 (54), 327 (21)
	4'-hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
	Temazepam	8.16	372	372 (28), 373 (10), 374 (12)
	Normedazepam			
Midazolam *(Versed)*	Hydroxymidazolam	9.12	413	413 (95), 310(100), 398 (88)
Nitrazepam *(Radedorm)*	7-Aminonitrazepam	7.63	395	394(100), 395 (96), 396 (40)
	7-Acetamidonitrazepam	7.96	437	436(100), 437 (98), 422 (20)
	2-Amino-5-nitro-benzophenone	7.03	314	299(100), 253 (30), 313 (15)
Nordiazepam *(Calmday)*	Unchanged	5.90	342	342 (64), 343 (54), 327 (21)
	4'-Hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
Oxazepam *(Serax)*	Unchanged	6.60	430	429(100), 430 (68), 431 (70)
Prazepam *(Centrax)*	Nordiazepam	5.90	342	342 (64), 343 (54), 327 (21)
	4'-Hydroxynordiazepam	8.12	430	429(100), 430 (68), 431 (70)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
	3-Hydroxyprazepam	9.30	412	383(100), 385 (40), 412 (33)
Temazepam *(Normison)*	Unchanged	8.16	372	372 (28), 373 (10), 374 (12)
	Oxazepam	6.60	430	429(100), 430 (68), 431 (70)
Triazolam *(Halcion)*	Hydroxytriazolam	11.53	430	415(100), 430 (60), 432 (40)

The benzodiazepine extraction procedure developed in this study is similar to that we apply routinely for conjugated steroids (30) with some modifications: the use of less concentrated of acetonitrile (15%) in a clean up stage; this is crucial for adequate recoveries. An essential element of the derivatization procedure is use of 1% TMCS as a catalyst. Without a catalyst, reaction is slow and yields multiple products for one compound. GC column stability is also an issue for routine testing. Column deterioration due to relatively dirty samples becomes a problem and results in peak tailing and irreversible adsorption for the most demanding polar compounds: oxazepam, lorazepam, 7-aminoclonazepam, 7-aminonitrazepam, and 4'-hydroxynordiazepam. As a result, GC column needs replacement every two weeks, when instrument runs samples routinely around the clock.

Common metabolites for many drugs in Table 1 make it difficult to identify the parent drug being administered. The diagram below shows conversion of individual drugs into the same metabolites, and into characteristic individual metabolites for some of them:



Specific pattern of urinary metabolites may help to distinguish between drugs (31). Diazepam and its major metabolites are shown in Fig. 2. The simultaneous presence of four metabolites, oxazepam, temazepam, 4'-hydroxynordiazepam and/or nordiazepam indicates administration of diazepam or one of its precursors. 4'-hydroxydiazepam is usually minor and may not be present. The combination of temazepam and oxazepam only indicates temazepam as a parent. Absence of temazepam is an indication of nordiazepam ingestion or its precursors. In the later stages of excretion when total amount of metabolites drop below 50 ng/mL, it may be difficult to determine the parent drug, since only oxazepam remains in the urine. Similarly, the parent drug may be determined for lorazepam related benzodiazepines, as shown in Fig. 3.

The other drugs in Table 1 do not have common metabolites and are easy to identify.

A chromatogram of our benzodiazepine mixture is shown in Fig. 4. This is used for routine quantitative calibration in every batch of urine samples along with controls and negative. For metabolites which are not available commercially and cannot be quantified, corresponding excretion urines are run for confirmation; this applies for bromazepam, clobazam and midazolam.

The frequency of detection of different drugs in routine is as follows from high to low:
Chlordiazepoxide, Diazepam, Clorazepate, Lorazepam, Alprazolam, Clonazepam, Midazolam,

Flurazepam, Temazepam, Triazolam, Flunitrazepam, Bromazepam.

GC/MS confirmation depends on the drug(s) found in sceen and usually includes the full metabolic pattern of a particular drug for reliable identification.

This procedure for expanded benzodiazepineshas been in routine use in our laboratory for several years for clinical and drug of abuse testing, especially in monitoring healthcare professinals.

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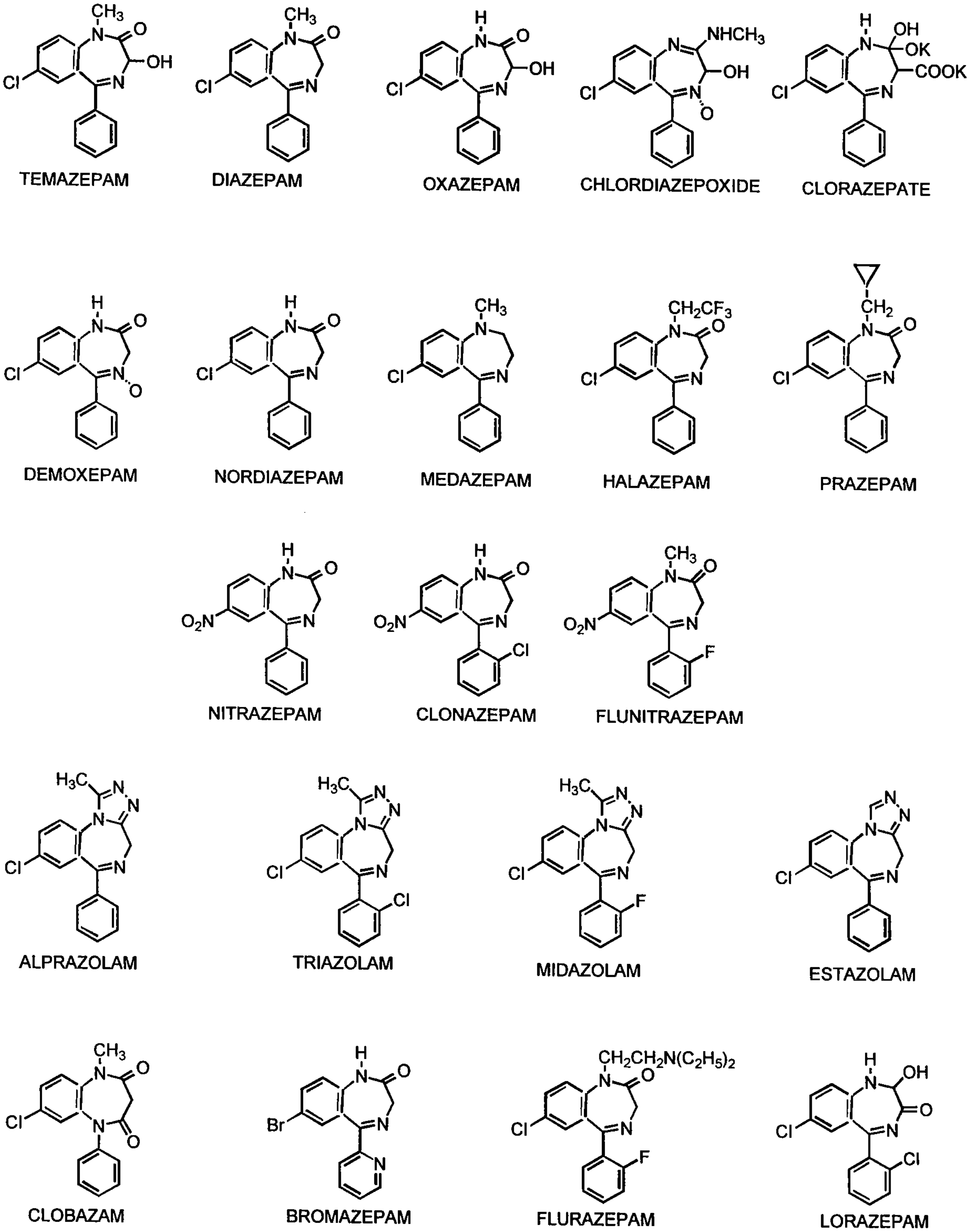

Figure 1. Structures of benzodiazepines.

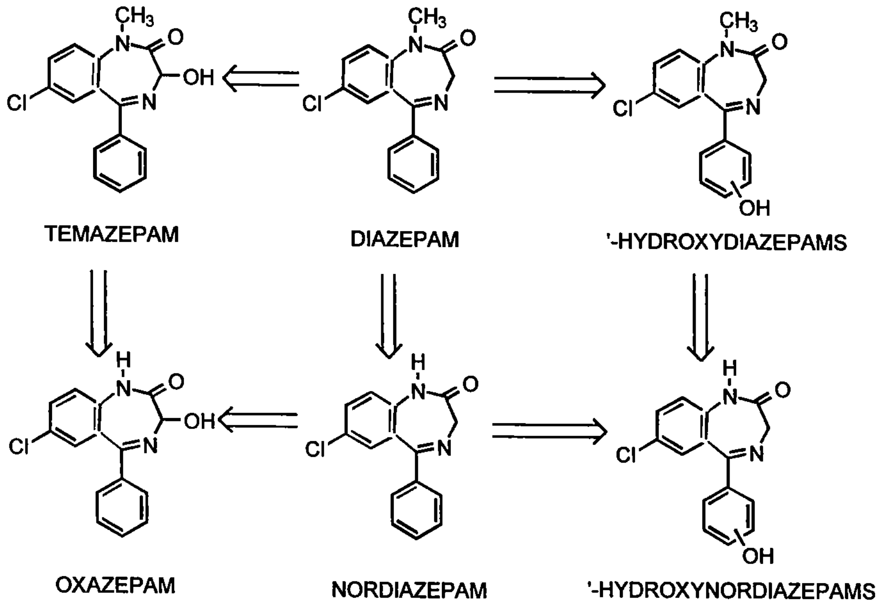

Figure 2. Metabolism of diazepam, temazepam and nordiazepam.

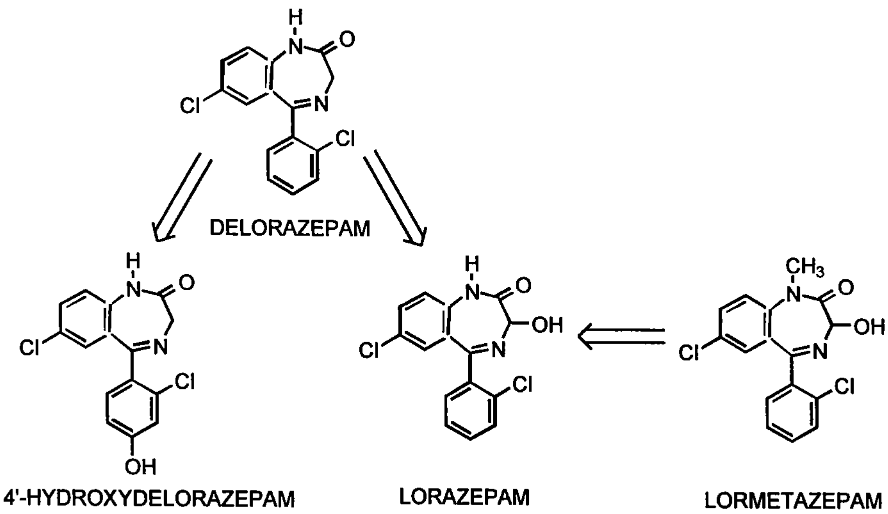

Figure 3. Metabolism of lorazepam analogs.

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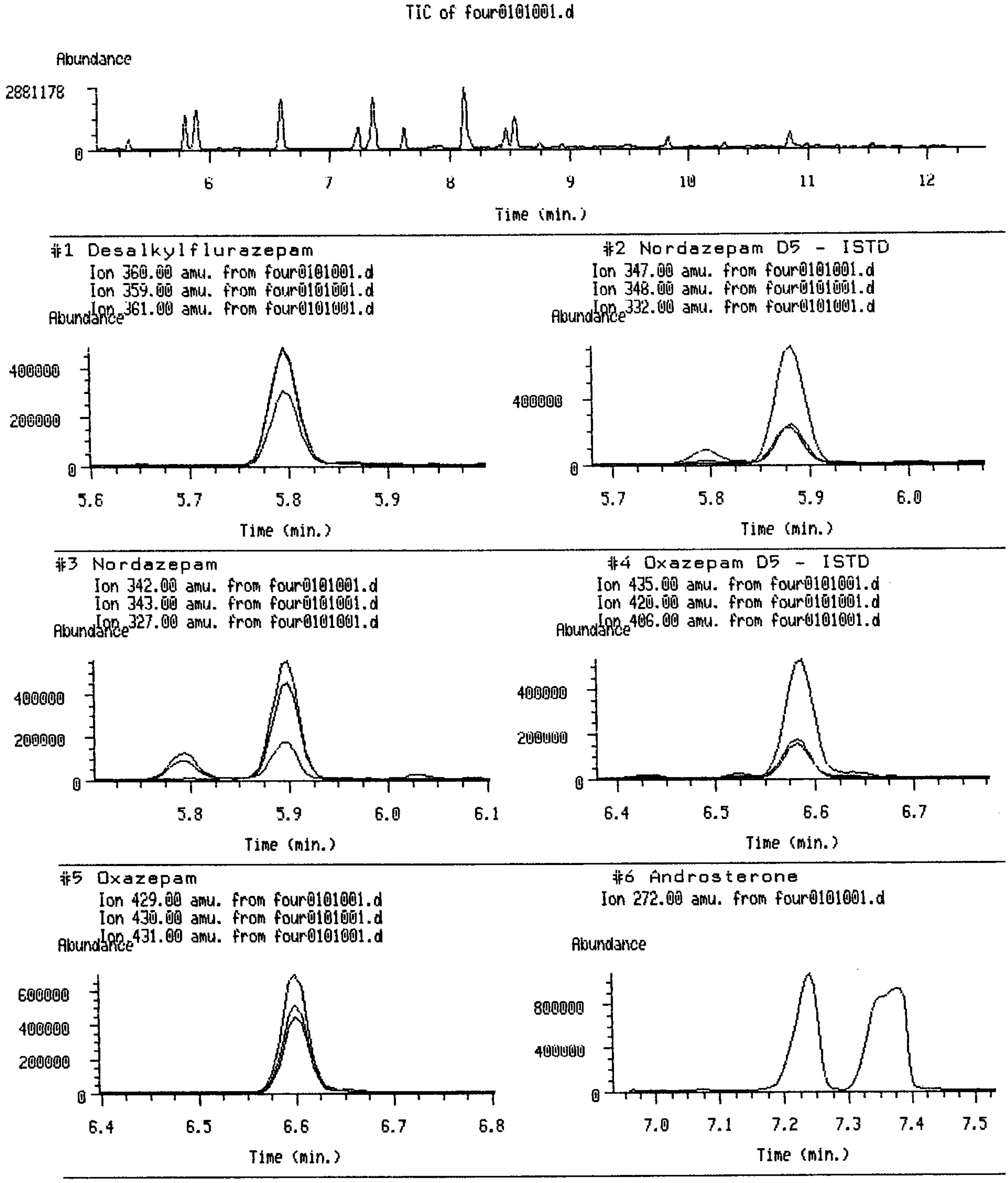

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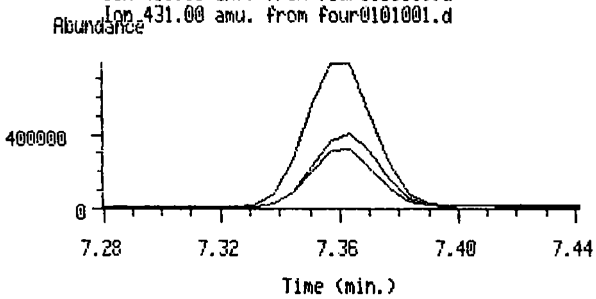
Ion 431.00 amu. from four0101001.d



#8 2',3'-OH-Nordazepams
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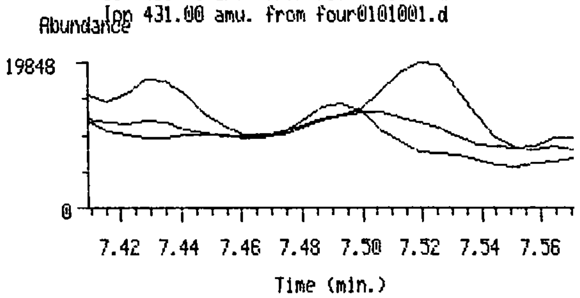

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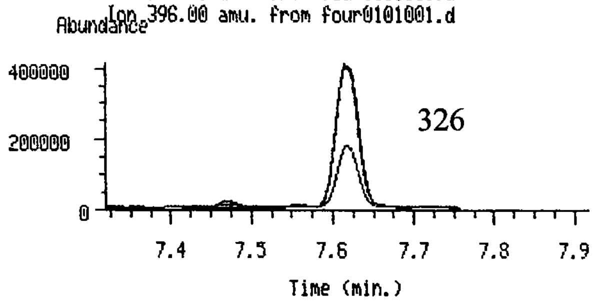

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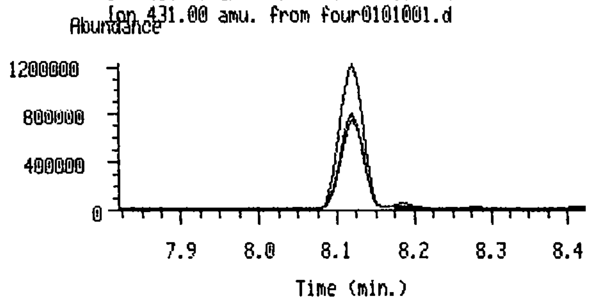

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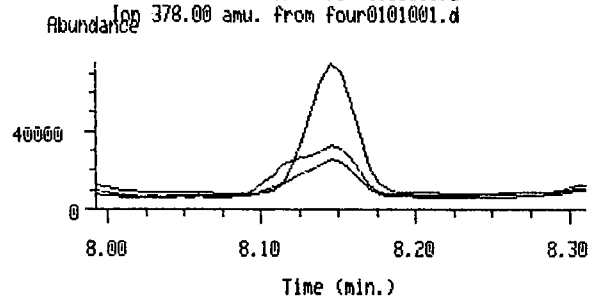

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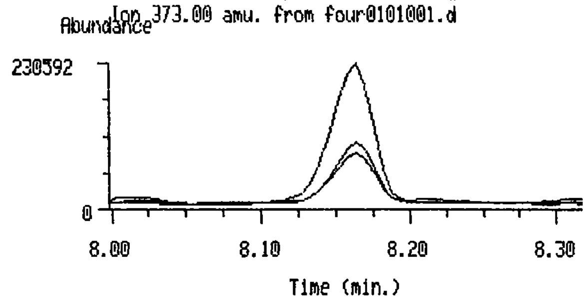

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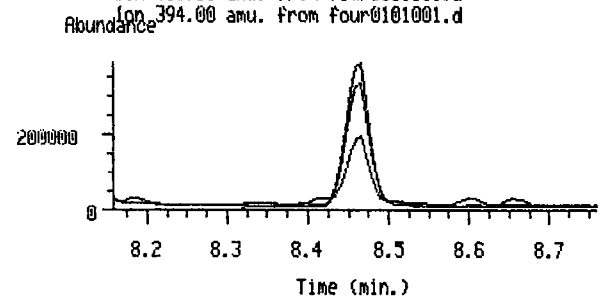

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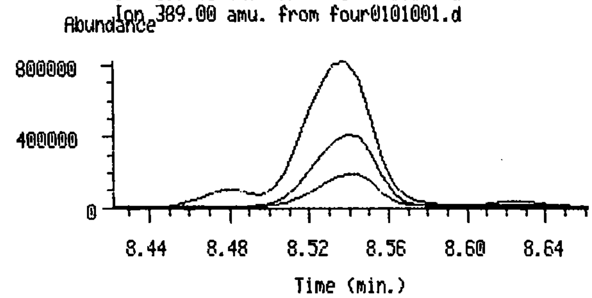

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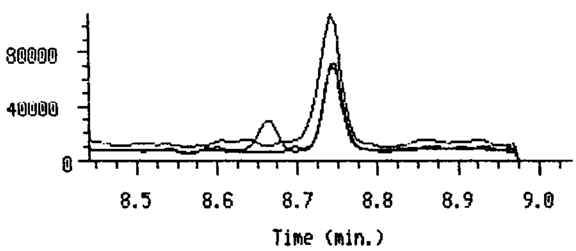

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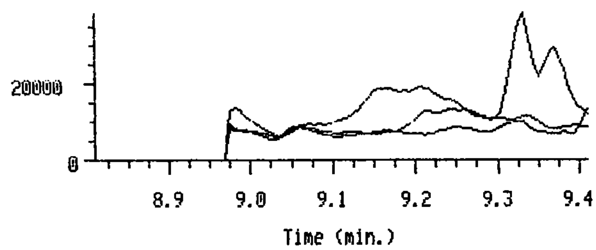

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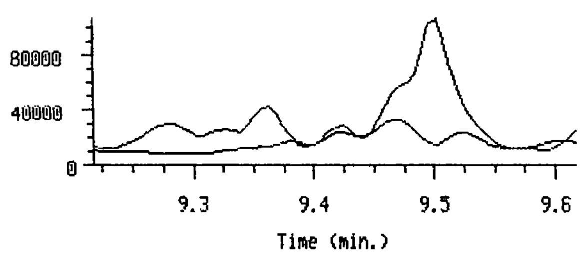

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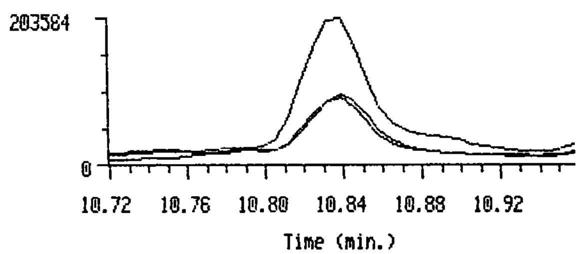
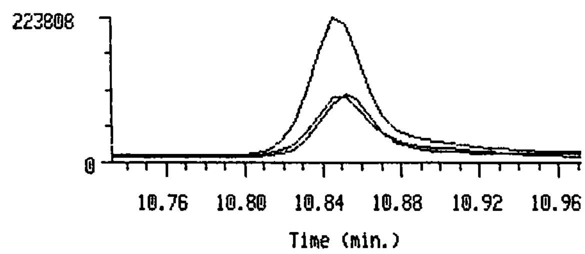
Ion 401.00 amu. from four0101001.d
Ion 388.00 amu. from four0101001.d



#19 OH-Alprazolam
Ion 381.00 amu. from four0101001.d
Ion 396.00 amu. from four0101001.d
Ion 383.00 amu. from four0101001.d



#20 OH-Triazolam
Ion 415.00 amu. from four0101001.d
Ion 430.00 amu. from four0101001.d
Ion 432.00 amu. from four0101001.d