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Confirmation of urinary benzodiazepines by gas chromatography/mass spectrometry

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CONFIRMATION OF URINARY BENZODIAZEPINES BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY

by

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ABSTRACT

A new method is described for the quantitative analysis of urinary benzodiazepines by gas chromatography/mass spectrometry. Development work was aimed at satisfying federal requirements for methods used in forensic urine drug testing which have become the standard in the laboratory industry.

Trimethylsilyl (TMS), tert-butyl-dimethylsilyl (TBDMS) and benzophenone derivatives were tested in the development of the new assay. TBDMS derivatives were found to be the most suitable for the analysis of six common benzodiazepine metabolites. Precison for all metabolites tested, as measured by the within run coefficient of variation, was less than 7% at 100 ng/ml (n=15). Assay sensitivity varied with the specific analyte in the range of 5 to 10 ng/ml. Validation of the procedure included the reanalysis of benzodiazepine positive urine specimens obtained from a forensic drug testing laboratory and comparison of the results from the independant assays. These specimens were tested first by radioimmunoassay using a 100 ng/ml cutoff and then confirmed by GC/MS. Sensitivity was sufficient to confirm the presence of benzodiazepine metabolites in all specimens tested.

INTRODUCTION

Benzodiazepines and Employee Drug Testing

Chlordiazepoxide was introduced into medical practice in 1960 as the first of a new class of drugs, the benzodiazepines. Since then, approximately two dozen benzodiazepines have been approved for use as anti-anxiolytic, sedative, anticonvulsant and muscle relaxant agents. Because these drugs are relatively safe and show few serious side effects, they have received wide acceptance in the medical community. A significant abuse potential has been attributed to many of these compounds, however, and they are often found to be contributing factors in automobile and industrial accidents. Consequently, the benzodiazepines are of interest to forensic toxicologists who are often asked to identify these drugs in biological specimens.

At the present time, there is a great demand for laboratories involved in employee drug testing, including the testing for benzodiazepines, to deliver results with a high degree of precision and accuracy. The necessity for this is evident when one considers the consequences to a job applicant who has been incorrectly identified as a drug user. Employer's are increasingly using the urine drug screen as one tool to assess an applicant's fitness for employment. This is particularly true in the transportation industry where public safety is the overriding concern. There is however, a question as to whether the technical capability exists to provide the necessary test accuracy for the benzodiazepines.

Responding to the demand for accuracy, laboratories have largely adopted standardized methodologies for the commonly abused drugs that

have been found to be essentially error free. These tests usually involve screening the urine with a rapid but relatively nonspecific immunochemical assay such as enzyme immunoassay (EIA) or radioimmunoassay (RIA). Positive results are confirmed by reanalysis using a highly specific technique such as GC/MS. Although sensitive immunochemical screening methods are available for the benzodiazepines, the literature provides few references for the quantitative GC/MS confirmation of this class of compounds. Difficulties inherent to the analysis of these compounds are largely responsible. As a result, confirmations offered by most laboratories are limited to only one or two compounds. Based on surveys of prescription benzodiazepine sales, it is expected that many immunochemical screen positives will not be confirmed. The need for improved GC/MS methodology is apparent.

Pharmacology and Toxicology

Over 2000 benzodiazepine compounds have been been synthesized and about two dozen have been introduced into clinical practice primarily for use as sedative-hypnotic agents (Gilman, et al. 1980). The most important members of this class, based on prescription use, are found in Table 1. Use of these drugs results in sedation at lower doses which progresses to hypnosis and stupor as the dose is increased. This is typical of general CNS depressants. Benzodiazepines have also been shown to be useful as muscle relaxants and in the treatment of certain forms of epilepsy. These primary effects are mediated by an action on the central nervous system. Unlike many other classes of CNS depressants including anesthetics, barbiturates and others, the

benzodiazepines are not general neuronal depressants since their activity is associated with an action on specific receptor sites and structures within the CNS. These compounds are also characterized by low toxicity. Death resulting from overdose is rare and because of their safety, various benzodiazepines have largely replaced the barbiturates as "sleeping pills" since their introduction in the sixties.

The evidence to date indicate that benzodiazepines act almost entirely by inhibition of the polysnaptic pathways within the CNS. The mechanism is primarily presynaptic and seems to be in some way related to the action or metabolism of the inhibitory neurotransmitter gamma amino butyric acid (GABA). Inhibitors of GABA synthesis prevent the action of benzodiazepines and inhibitors of GABA degradation augment their action. These and other observations suggest that benzodiazepines function indirectly to modify the action of GABA. One hypothesis proposed is that benzodiazepines may act to antagonize a protein that inhibits the binding of GABA to its receptor (Gilman, et al. 1980).

A number of undesirable side effects are associated with the use of these drugs. These include impairment of reaction time and coordination which is potentiated by the use of alcohol. Because of this, prescriptions are accompanied by a warning against the operation of motor vehicles. Other side effects include various sleeping disorders including vivid nightmares. Amnesia is also common. Tolerance and dependence has been reported in individuals using these compounds over an extended period of time. Withdrawal of the drug from the dependent individual may precipitate a withdrawal syndrome similar to barbiturate

withdrawal (Costa 1983). Although habit forming, addiction is not common and illicit use may be more often associated with attempts to mitigate the undesired side effects of illicit drugs such as amphetamines and cocaine. One group with a significant risk of dependence are alcoholics receiving chlordiazepoxide to aid in the withdrawal of alcohol (Costa 1983, Gerd, et al. 1984).

Pharmacokinetics and Metabolism

Benzodiazepines are usually administered orally in doses of 0.5 to 30 mg depending on the specific drug. Adsorption is rapid with most preparations and the effects of the drug are experienced within an hour of ingestion. Following adsorption, blood levels peak in the low nanogram to low microgram per milliliter range, again depending on the dose and compound administered. Because the various drugs differ considerably in their metabolism and pharmacokinetics, it is difficult to characterize them as a class.

The metabolic pathways for each of the commonly prescribed benzodiazepines are illustrated in Figures 1-3. The reactions illustrated, namely hydroxylation and dealkylation followed by glucuronide conjugation, occur primarily in the liver. Hydroxylation is common at the three position leading to the formation of 3 hydroxy metabolites. Glucuronide conjugation follows at the same position. A number of benzodiazepine compounds follow a metabolic pathway leading to oxazepam and/or nordiazepam as final end products. Examples are diazepam, chlordiazepoxide, temazepam, prazepam and medazepam (Baselt 1982, Costa 1983). The metabolism of certain other benzodiazepines

results in the production of end products unique to the parent drug. Examples are lorazepam, flurazepam, alprazolam and triazolam.

Chlordiazepoxide was the first drug of the class introduced into clinical practice. Compounds introduced later often represent modifications to the basic structure of this drug. Much of the action of the drug is produced by a number of pharmacologically active metabolites that appear in the blood soon after adsorption. These include oxazepam, demoxepam, nordiazepam and norchlordiazepoxide. The combined therapeutic range of chlordiazepoxide and it's active metabolites is 0.4 to 4.0 mcg/ml (Moffat 1986). With the exception of oxazepam, these metabolites have longer half lives than the parent drug and tend to accumulate in blood with repeated administration (Greenblatt, et al. 1978, Guentert 1984). Pharmacokinetics are consistent with a two-compartment model although a three-compartment model has also been proposed based on the lipophilic nature of the drug and its potential to accumulate in adipose tissue. The half live of one of the metabolites, nordiazepam, has been determined to be 40-100 hours. Assuming 5 half lives are required for elimination of the drug and the half life in an individual is 100 hours, it follows that 500 hours (21 days) would be required to eliminate a single dose of the drug. Most of the drug is eliminated in the urine as glucuronide conjugates of oxazepam and nordiazepam.

Diazepam has a number of pharmacokinetic characteristics in common with chlordiazepoxide. Also lipophilic, metabolism results in the formation of the active metabolites oxazepam, nordiazepam and to a small extent, 3-OH diazepam. Therapeutic levels for the sum of diazepam and

nordiazepam are in the range of 0.5 to 1.0 mcg/ml. Nordiazepam tends to accumulate in plasma with repeated administration. Like chlordiazepoxide, elimination is prolonged, particularly with repeated administration of the drug. In a study involving chronic use, both diazepam and nordiazepam were detectable in the blood 7 days after the drug was discontinued (Moffat 1986). Diazepam is eliminated primarily in the urine as glucuronide conjugates of oxazepam, nordiazepam and to a lesser extent, 3-OH diazepam (Baselt 1982, Marinella, et al. 1978).

Flurazepam is another of the lipophilic benzodiazepines. Unlike diazepam and chlordiazepoxide however, very little of the action of the drug is due to the parent compound. Following adsorption, flurazepam is almost entirely metabolized to active metabolites on its first pass through the liver. As a result, very little or no flurazepam is detectable in blood with normal use. Metabolism precedes along two pathways. The first leads to the formation of an important active metabolite, desalkylflurazepam with a half life of 2-5 days. The therapeutic range for flurazepam is 30-150 ng/ml, defined in terms of this active metabolite. Like diazepam and chlordiazepoxide, "hangover" following use of the drug is common and attributable to the delayed elimination of active metabolites (Greenblatt, et al. 1975).

A second metabolic pathway leads to the formation of another active metabolite, N-1-hydroxyethylflurazepam with a half life of 10-20 hours. It is detectable in the blood at low concentrations for only a short period post dose and probably does not contribute very much to the overall effect of the drug. Flurazepam is eliminated primarily in the urine as the glucuronide conjugate of HO-ethylflurazepam, representing

29%-55% of the dose. Smaller concentrations of conjugated N-1-desalkyl-3-hydroxyflurazepam are also detected. Elimination is reported to be essentially complete 72 hours post dose. This figure is difficult to reconcile, however with the half life of two to five days reported for the N-desalkyl metabolite. It seems likely that the elimination time reported for many drugs apply mostly to single dose studies and may not reflect accumulation of lipophilic metabolites.

Temazepam (3-OH diazepam) and oxazepam are both active metabolites of diazepam and are prescribed as pharmacological agents in their own right. The half lives of temazepam and oxazepam are 10 and 8 hours, respectively. Elimination of both compounds is rapid and essentially complete in 72 hours. Both compounds are eliminated as the glucuronide conjugates of the unchanged drugs. Lorazepam is a minor variation of the structure of oxazepam containing a chlorine at position 2 of the 5 phenyl ring. Pharmacokinetics including elimination are quite similar to oxazepam (Greenblatt 1981, Heel, et al. 1981).

The triazolobenzodiazepines, alprazolam and triazolam, are administered orally in doses of 0.5 to 3 mg. These relatively low doses result in correspondingly low plasma levels in the low ng/ml range. Both compounds are extensively metabolized by hydroxylation leading to the formation of approximately 20 metabolites. Many of these are active, but none achieve significant plasma levels and do not contribute to the activity of either drug. The half life of both drugs is on the order of 10 hours and elimination is complete at 72 hours. The pharmacokinetics of these compounds is similar to to other short half life benzodiazepines. Both drugs are eliminated primarily in the urine as

conjugates of the hydroxylated metabolites. Most important of these is alpha hydroxyalprazolam and alpha hydroxytriazolam which represent about 17% of the total dose. Other important metabolites include the 4-hydroxy metabolites and a hydroxylated benzophenone (Moffat 1986, Dawson, et al. 1984, Houts, et al. 1988).

Federal Regulation and Methodology Used in Employee Drug Testing

Methodology used by laboratories for employee drug testing has become increasingly standardized to conform to the Mandatory Guidelines for Federal Workplace Drug Testing Programs as set forth in the Federal Register, April 1988. In order for laboratories to participate in the federal drug testing program, they must be accredited by the National Institute of Drug Abuse (NIDA). This new accreditation program closely resembles the program used to accredit Department of Defense (DOD) laboratories responsible for the testing of all armed services personal. The experience of these laboratories in the testing of millions of specimens, and in legally supporting the results, is reflected in the new federal guidelines. The test results produced by these laboratories is considered second to none.

NIDA accreditation requires that laboratories comply fully with the guidelines, successfully complete three rounds of proficiency testing and pass a rigorous inspection. Once accredited laboratories must demonstrate a continued level of proficiency by means of open and blind proficiency testing. The quality control requirements of this program are far more demanding than those in general use within private industry. For example, to maintain accreditation, laboratories must be

able to correctly identify drugs contained in blind specimens and quantitate to within 20% of the spiked value. Loss of accreditation results automatically with the reporting of a false positive in either a real or proficiency specimen. The quantitative accuracy and precision required by this program in many cases severely challenges GC/MS drug testing methodology and it is this aspect of the program that perhaps presents the most difficulty for laboratories seeking accreditation. Private laboratories are rushing to bring themselves in compliance with the standards for accreditation as set forth in the Federal Register because, in effect, laboratories with NIDA accreditation will be the "Gold Standard" against which all other laboratories are measured.

Under the new federal guidelines the testing protocol requires that specimens be tested first by an immunochemical technique and if positive, confirmed by quantitative GC/MS. The gas chromatograph/mass spectrometer is capable of acquiring data in two modes. The first is SCAN mode in which the complete mass spectra for compounds eluting from the gas chromatograph are acquired. The second is selected ion mode or SIM in which only selected ions of the eluted compounds are acquired. In order to obtain quantitative results, the mass spectrometer must be operated in SIM.

Both methods of acquisition are used in the identification of drugs. The scan mode has the advantage in providing the complete mass spectrum which can be computer matched to stored mass spectral libraries. A great deal of information can be contained in a single EI mass spectrum which in turn greatly enhances the specificity of the

analysis and the operator's confidence in identification. The reliability of scan results will be somewhat dependent on the skill, judgment and experience of the operator. At lower analyte concentrations the quality of mass spectra declines and at these lower concentrations misidentifications are more likely. The low concentrations at which drugs are detected by immunochemical methods often precludes the use of SCAN for confirmation.

When operated in SIM, three characteristic ions for each compound are typically acquired. The most abundant of the three is used for quantitation and the other two as "peak qualifiers." These ions are generated by the MS in a reproducible fashion so that the ratio of these qualifier ions to the quantitative ion can be used to add specificity to the identification. Sensitivity is greatly improved in SIM in part because of an improved signal to noise ratio. Since only a few characteristic ions are monitored, the co-eluting compounds seen as chromatographic noise in SCAN are not seen. Consequently, confirmation in the low ng/ml range is possible. Deuterium labeled analogues of many analytes are available for use as internal standards greatly enhancing the precision of SIM quantitations.

NIDA and the DOD chose the quantitative SIM method of drug confirmation primarily for two interrelated reasons. First only SIM can consistently provide the sensitivity required to confirm the positive results generated by immunochemical techniques. The second reason is that SIM methodology lends itself far more easily to the application of objective criteria for the determination of test results. If a consensus among experts can be obtained for these criteria, then it is

far less likely that test results will be successfully challenged based on the methodology used. These criteria used to find a specimen positive include, but are not limited to the following: (1) The specimen must have a quantitative value equal to or greater than a predetermined limit of analysis. (2) Qualifier ratios must correspond to the qualifier ratios of the standard used to calibrate the instrument. (3) Retention times for the drug and internal standard must correspond to those of the standard used in calibrating the instrument. Because results are determined based on a quantitative limit of analysis, the instrument is calibrated at this concentration for ion ratios, retention times and quantitation. For the same reason, precision of the assay at the limit of analysis as well as quantitative accuracy become important issues and both must be documented.

Screening and confirmation concentrations for the five NIDA drugs are set by NIDA. For some of the drug groups immunochemical results correspond fairly well to GC/MS confirmation results. An example is phencyclidine which has screening and confirmation limits of analysis both set at 25 ng/ml. For other drug groups the immunochemical screen positive may be the result of several metabolites present in the urine. The presence of cocaine is detected using a screening limit value of 300 ng/ml but when confirmed as one of it's metabolites, benzoylecgonine, it's limit is 150 ng/ml. In the development of a new confirmatory assay, the limit of analysis is determined experimentally to be a value that will reliably confirm a large percentage of immunochemical positives. This is done by correlating a large number of immunochemical positive urines with GC/MS results obtained on the same specimens. All

GC/MS confirmation assays are required to have a confirmation limit of analysis equal to or less than the screen limit.

At the present time, NIDA requires laboratories seeking accreditation to provide methodology for the screening and confirmation of five drug groups. These are the amphetamines, opiates, cannabis, cocaine and PCP. The five drug groups selected corresponds to those drugs currently tested by the armed forces. Methodology currently exists for these drugs, capable of meeting NIDA quality control requirements. However, NIDA also recognizes the need for the testing of other abused substances as evidenced in NIDA research monograph 73. This publication is intended to provide information to those organizations considering the implementation of a drug testing program. Included are sections dealing with drug pharmacology, quality control and proficiency testing, specimen collection and handling and a section summarizing current analytical methodology for the most commonly abused drugs. In addition to the five drug groups currently required by NIDA, the monograph contains a summary of methodology and pharmacokinetics for methaqualone, LSD, alcohol, barbiturates and benzodiazepines. Interestingly, none of the references in the benzodiazepine section provided the quantitative GC/MS methodology required by NIDA. A more thorough literature search, indicates that in fact such proven methodology does not exist, at least not in the public domain.

The most commonly used immunoassays are the EMIT assay, the trademark for the EIA produced by the Syva Corp., and the Abuscreen, the trademark for the RIA produced by Roche Diagnostics Corp. These assays have limits of analysis of 300 and 100 ng/ml, respectively. An

accreditable GC/MS assay must confirm the presence of benzodiazepines at these levels and be capable of quantitative accuracy within 20% of the known value at these limits. This will require the assay to provide precision, as measured by the between run coefficient of variation to be less than 10% at the limit of analysis. Deuterated internal standards for two benzodiazepine metabolites, oxazepam-D5 and nordiazepam-D5 are available. The required precision for at least these two compounds should be possible.

Quantitative GC/MS requires the operation of the instrument in selected ion mode which in turn requires the operator to select in advance those compounds to be confirmed. It is probably impractical to look for the dozens of compounds and metabolites now on the market so it is necessary in the design of the assay to select the most commonly prescribed compounds which presumably would also be most likely to be present in a urine specimen. Based on recent surveys of prescription benzodiazepine sales, the primary urinary metabolites of the compounds listed in Table 1 are likely candidates for inclusion in a confirmatory assay.

Problems Relating to the GC Analysis of Benzodiazepines

Difficulties in the GC/MS analysis of benzodiazepines are related to the multiplicity of the compounds to be tested and the chemical nature of the drugs. Some of these compounds, particularly oxazepam, undergo thermal degradation in the gas chromatograph. All of these metabolites have polar hydroxyl or amino groups making gas chromatograpic analysis of the underivatized drug difficult. Liquid

chromatographic methods work well for these compounds, and based on their chemistry, LC/MS should be the confirmation method of choice. Unfortunately, the technology is still fairly new and the instrumentation is prohibitively expensive for most commercial laboratories. In addition, GC/MS is the only NIDA approved method of confirmation.

Fortunately, benzodiazepines can be hydrolyzed or derivatized to less polar and more stable compounds more amenable to GC/MS analysis. When heated at low pH, many benzodiazepines are hydrolyzed to benzophenones, compounds with improved chromatographic properties over the parent drugs. Benzophenones and can be further derivatized with various acetylation reagents such as pentafluoropropionic anhydride resulting in additional gains in chromatographic performance. In the MS these derivatives form abundant, high molecular weight ions, ideal for this kind of analysis. Acid hydrolysis of benzodiazepines to benzophenones has a disadvantage in that many benzodiazepines form the same benzophenone. The acid hydrolysis of a urine specimen containing oxazepam, nordiazepam and norchlordiazepoxide would yield a single benzophenone, 2-amino-5-chlorobenzophenone (ACB). Another benzophenone of interest is 2-methylamino-5-chlorobenzophenone (MACB) formed by the hydrolysis of diazepam and 3-OH diazepam (temazepam). Other metabolites, including HO-ethylflurazepam and lorazepam can be hydrolyzed to benzophenones unique to their parent compound. Identification of these benzophenones allows positive identification of the drug used. The useful chromatographic properties of benzophenones have been exploited in many qualitative GC and GC/MS procedures. Unfortunately not all benzodiazepines form benzophenones. Examples of ones that do not are

the urinary metabolites of alprazolam and triazolam.

A number of other derivatization reactions, including silylation, methylation and acylation, have been devised to overcome the problems associated with GC analysis. Add to these the use of benzophenone derivatives, and the chemist has a number of alternative approaches to the analysis of these compounds. In the development of this methodology the selection of a common derivatization scheme for this rather diverse class of compounds becomes critical.

Analytical Methods for Benzodiazepines

The papers reviewed in this section are representative of the analytical methodology used in the the gas chromatographic and gas chromatographic/mass spectrometric determination of benzodiazepines. Early published methods were primarily intended for use in developing pharmacokinetic data for the drugs and metabolites in blood. Gas chromatography was favored because it was found to be both highly selective and sensitive, especially using electron capture detection. The extraction and derivatization procedures found in these papers are often applicable to the quantitative confirmation of these drugs in urine. A smaller number of papers describe methodology specifically intended for the identification of benzodiazepines in urine. In most cases these methods were developed as qualitative techniques for use in clinical or forensic drug testing laboratories. The convention of using quantitative confirmatory techniques in urine drug testing is fairly new, and references for this kind of analysis are recent.

A number of other analytical techniques have been developed for the

analysis of these drugs. High performance liquid chromatography has been found especially useful for pharmacokinetic work and in therapeutic drug monitoring. Although not as sensitive as GC/ECD, HPLC has the advantage of allowing the accurate analysis of benzodiazepines without derivatization. In general, however, methods other than GC and GC/MS, have not found wide acceptance in drug testing labs and will not be reviewed here.

Some of the earliest and most detailed work on benzodiazepine methodology and pharmacokinetics was done by Arthur F. de Silva, et al. at Hoffman-La Roche. In a paper published in 1970 he describes extraction and GC/ECD procedures applicable to blood or urine for medazepam and it's metabolites, diazepam, nordiazepam, temazepam and oxazepam. This early procedure involved extracting the sample twice with diethyl ether, combining the extracts and then back extracting the ether with 2.0 N HCl. The ether is removed and discarded, the aqueous solution is made basic with 2 N NaOH and extracted again with diethyl ether. This final ether extract is then evaporated and the underivatized drugs injected on the GC/ECD using OV-17 as the stationary phase. The methodology is used to generate pharmacokinetic data in subjects using the drug. Sensitivity of the method is approximately 50 ng/ml for all compounds tested in blood or urine. Somewhat surprising is the fact that good sensitivity and linearity is obtained by this author without derivatization, although he uses a TMS derivative for 3-OH diazepam in special circumstances. Precision data is not reported. In addition to the blood pharmacokinetic data, some limited urinary excretion data was generated to show the percentage of the dose eliminated by this route.

In 1976 the same author published in a single paper extraction, derivatization and GC/ECD procedures for essentially all benzodiazepines and their metabolites in blood. These new procedures were somewhat simpler, less time consuming and resulted in improved sensitivity over earlier work. He found that 20 benzodiazepine compounds tested could be extracted optimally by one of three simplified methods. These methods involved the direct extraction at alkaline pH with diethyl ether or protein precipitation of the sample followed by extraction with benzene/ methylene chloride (90:10). Extracts were derivatized to form either the TMS derivatives, methylated derivatives or benzophenones depending on the compound of interest and quantitated on GC/ECD using OV-17 as the stationary phase. The author recommends the use of either TMS or benzophenone derivatives for lorazepam, oxazepam, and temazepam. Demoxepam, desalkylflurazepam, clonazepam and others are tested as their methyl derivatives. Mention is made of the fact that derivatization of thermally unstable benzodiazepines is necessary for good chromatography and consistent results. Precision and linearity data for these methods are not reported.

NIDA Research Monograph 73 (1986) cites seven papers in its section under benzodiazepines. These cover a variety of techniques for the analysis of these compounds including EMIT, TLC, HPLC and GC/NP but none described methodology for GC/MS determination of these compounds. One paper cited in the monograph by Karl Verebey, et al. (1982) describes the GC/NP confirmation of benzophenones and it's application to forensic urine drug testing. The methodology described in this paper turned out to be quite useful and became the starting point for the MS work to be

described in later sections of the thesis.

The Verebey assay begins with an acid hydrolysis which serves two purposes. First the hydrolysis frees urinary conjugates and second, it serves to form benzophenones, both in the same step. The hydrolyzed urine is then made basic, extracted with hexane and the extract injected on GC/NP for the identification of the benzophenones. This procedure is simple and quick. Because hexane is a non-polar solvent, extracts of urine hydrolysates are very clean. The author used this method to identify Valium (diazepam) use by the identification of the benzophenones: aminochlorobenzophenone (ACB) and methyl-aminochlorobenzophenone (MACB).

In the same paper, (Verebey, et al. 1982) the urinary excretion profiles of diazepam, as measured by the quantitation of ACB and MACB were determined in three subjects receiving 10 mg diazepam once a day for five days. Urines were collected on each of the five days the drug was received as well as seven days after the drug was discontinued. The same urines were also tested by EMIT. Specimens obtained during the first two days of administration were often negative. One subject remained negative until after four consecutive days of use. Concentrations of the drug were generally highest on the last day the drug was given and remained EMIT positive for days after the drug was discontinued. One subject was EMIT positive on the last collection day, seven days post dose. It was suggested that the obesity of this subject was the probable cause of the delayed elimination of the drug. Other subjects with normal body fat tested negative by EMIT before the seventh day post dose, although benzodiazepine metabolites were detectable in

these subjects by GC/NP at lower levels. The data suggests that elimination of the drug requires at least one week and is subject to substantial individual variation.

A paper by Baselt and co-workers (1977) described the analysis of underivatized benzodiazepines in body fluids by GC/FID. Mention was made of the fact that benzophenone derivatives lack specificity since they result from the hydrolysis of many different benzodiazepine compounds. Having pointed out this difficulty, Baselt described his own protocol for the analysis of the underivatized drugs. This involved a liquid/liquid extraction with back extraction into acid and finally GC/FID quantitation using OV-17 as the stationary phase. Although a number of benzodiazepines could be quantitated by this approach, the method suffered from poor precision, particularly for oxazepam and chlordiazepoxide, which are both thermally unstable. Linearity was established over the range of 200 to 2000 ng/ml. The author also cautioned that results should be confirmed with a second method since the method was subject to interference by many compounds.

Despite obvious interpretative problems related to the analysis of benzophenones, the advantages in terms of improved chromatography resulted in continued interest and numerous publications. Relevant to urine drug testing is a paper by Maurer and Pfleger (1980). In this paper, the authors determined the GC retention indices and collected the MS spectra for most benzophenones. The GC/MS procedure described was qualitative in nature and provided no information on detection limits or linearities of the compounds detected. In many cases it was able to identify the parent compounds ingested from the urinary benzophenones

detected.

Benzophenone derivatives were also found useful in the quantitative analysis of certain benzodiazepines in blood. A method published by Sanchez, et al. (1976) involved hydrolyzing blood extracts to form the benzophenone derivative and then reacting the benzophenone with trifluoroacetic anhydride to form a benzophenone trifluoroacyl derivative. The author found the acyl derivatives had improved chromatographic properties over the underivatized benzophenones. Using this technique, Sanchez was able to detect as little as 5 ng/ml pivoxazepam in 3 ml plasma.

Joyce, et al. (1984) succeeded in documenting the thermal decomposition of benzodiazepines in the gas chromatograph. In this work a variety of benzodiazepines were injected into a GC/MS and the spectra generated were compared to those obtained by means of a direct insertion probe. The spectra obtained by these two techniques were quite different for oxazepam, chlordiazepoxide, demoxepam, temazepam, lorazepam and other thermally unstable benzodiazepines. Additionally, some benzodiazepines including clonazepam, that were thought to be stable, produced a decomposition product in the GC/MS that was seen as an additional peak. The effect was found to be concentration dependent in that higher concentrations of drug injected resulted in less decomposition when expressed as a percentage of the injected amount.

More recently, analytical procedures for a new class of benzodiazepines, the triazolobenzodiazepines, have appeared in the literature. These compounds are unlike the compounds already discussed in that they are not hydrolyzed under mild acid conditions and do not form

benzophenones. The most important compounds of this class in terms of use are alprazolam and triazolam. Joern, et al. (1987) described a method for the quantitative confirmation in urine of alprazolam, alpha hydroxyalprazolam and 3-hydroxymethyl-5-methyltriazolyl-chlorobenzophenone (3-HM) by GC/NP. The work of this author was directed specifically to providing information useful to toxicologists' involved in forensic urine drug testing. Documentation was provided on the precision and linearity of the assay as well as some urinary excretion data of the drug and metabolites in subjects receiving the drug.

The procedure was fairly straightforward, beginning with an enzyme hydrolysis of the urine specimen to cleave glucuronide conjugates, followed by extraction at alkaline pH with dichloromethane containing triazolam used as an internal standard. Because the alprazolam metabolites chromatographed poorly, extracts were derivatized with acetic anhydride to form the acyl derivatives. The derivatized extracts were then chromatographed by dual capillary GC/NP using OV-17 and SE-54 as the stationary phases. Peak shapes, linearity and precision were all quite good although retention times on the polar OV-17 column must be considered excessive (greater than 20 minutes) from a practical standpoint. The author found that concentrations of alpha-HA were highest between 13-23 hours following a single dose. Concentrations of 3-HM peaked at 2 hours post dose and were in general about one fourth the concentration of alpha-HA. Elimination was found to be essentially complete in 72 hours. Concentrations of these metabolites were also measured in hospitalized patients receiving the drug. Urinary levels in these patients were found to be two to four times higher for both 3-HM

and alpha-HA compared to the levels in the single dose study.

Another approach to the analysis of the triazolobenzodiazepines was found in an article by Coassolo, et al. (1983). The author was interested in developing GC methodology to be used in generating pharmacokinetic data for triazolam and the hydroxylated metabolites in blood and urine. Because a therapeutic dose of this drug results in very low plasma levels, a very sensitive method was required. Adequate instrument sensitivity was obtained using capillary GC with ECD detection. His procedure involved extraction of the sample at alkaline pH with diethyl ether with back extraction into acid. The acid was then made alkaline and re-extracted with diethyl ether. This final extract was evaporated and derivatized with BSTFA to form the TMS derivatives of the hydroxylated metabolites. Sensitivity was reported to be in the range of 0.2 ng/ml for all the compounds tested and precision, as measured by the coefficient of variation at 1 ng/ml, was 5%.

Mule, et al. (1989b) reported a method for the quantitative confirmation of benzodiazepines in urine by GC/MS. Six benzodiazepines were included in the method: oxazepam, nordiazepam, lorazepam, alpha-hydroxymidazolam, alpha-HA and alpha-HT. This selection of analytes was somewhat unusual based on the intended purpose of the assay: the confirmation of EMIT positive urines. Midazolam was available only as an I.V. preparation and would not be found outside a hospital setting. The assay was not designed to detect the use of temazepam or flurazepam, two very common compounds.

The procedure involves hydrolysis of the urine with the enzyme glucuronidase followed by extraction with dichloromethane and

derivatization with N, O-bis(Trimethylsilyl)trifluoroacetamide to form the trimethylsilyl derivatives. Linearity for oxazepam, nordiazepam and lorazepam over the range of 50 to 500 ng/ml was very good. Precision, as measured by the coefficient of variation, at 500 ng/ml was in the range of 5-10%. Although the method performed well for these compounds, problems were encountered in the analysis of the triazolobenzodiazepines. Linearities were not reported and within run precision for alpha-HA at the very high level of 2000 ng/ml was only 19%. Retention times for alpha-HA and alpha-HT were long (14 and 16 minutes, respectively) and both compounds chromatographed as asymmetric peaks. Mule found that the procedure was not sufficiently sensitive to confirm the presence of the alprazolam and triazolam metabolites at the concentrations expected in human urine.

With the implementation of the federal drug initiative there is now a defacto consensus among forensic toxicologists favoring quantitative GC/MS for the confirmation of abused drugs. These developments are recent, explaining the paucity of quantitative urine methods found for the benzodiazepines. The paper by Mule, et al. (1989b) is apparently the first quantitative GC/MS method for urinary benzodiazepines. Within the last year a few papers have been published for the quantitative confirmation in urine of other abused drugs. In many cases these are variations of long established methods that have been validated for the first time for quantitative accuracy and precision. An example is another recent paper by Mule, et al. (1989a) for the quantitative confirmation of barbiturates in urine by GC/MS. Documented in this paper are the linearity, precision and accuracy of the assay. The paper is

clearly directed to satisfying federal requirements for confirmatory testing.

From the literature surveyed, it is apparent that numerous approaches have been used in the analysis of this difficult class of compounds. Good accuracy and precision has been demonstrated in many assays, but these are specialized for the analysis of a limited number of compounds. To date, a comprehensive assay has not been reported to solve the problem at hand.

Applications for a Quantitative Urine GC/MS Assay

The object of this research was, in part, to develop a NIDA type GC/MS method for benzodiazepines. There was interest also in correlating RIA results of human specimens with GC/MS values for the various benzodiazepine metabolites. Studies correlating screening and confirmation methods are used primarily to asses the predicative value and reliability of the both methods and as a means of setting rational limits of analysis. These types of studies are appearing with greater frequency in the literature. Cone, et al. (1989b) correlated quantitative RIA, TDX, and EMIT results for cocaine positive specimens to GC/MS results for the same specimens. The degree of bias of the various immunochemical techniques was determined by plotting the value generated by the method against the GC/MS value. When plotted in this way, a straight line plot with a slope of 1.0 represents zero bias or perfect correlation.

Toxicologists are often asked to estimate the length of time a compound may be detected in the urine after a dose. The use of

published pharmacokinetic data including blood half lives are helpful, but may at times be misleading, particularly for lipophilic drugs that may be eliminated over a prolonged period of time. As a result, there is interest in determining the urinary excretion profiles for abused drugs. Cone, et al. (1989a) recently determined the urinary excretion profiles for cocaine in four experienced users after I.V. use. Joern, et al. (1987) performed a similar study for alprazolam in order to determine the concentrations of metabolites to be expected after use and the length of time they could be detected.

The proposed methodology could be used to test a large specimen population and determine what predicative value, if any is provided by surveys of prescription use. For example, it is seen that alprazolam is frequently prescribed, but whether it represents a problem in terms of abuse can only be answered if there is an analytical technique for the compound.

Table 1. Commonly Prescribed Benzodiazepines and Their Primary Urinary Metabolites.

	GENERIC	TRADE NAME	MAJOR URINARY METABOLITES
1.	Alprazolam	Xanax	alpha hydroxyalprazolam 4-hydroxyalprazolam 3-HM* alprazolam
2.	Diazepam	Valium	oxazepam nordiazepam 3-OH diazepam
3.	Triazolam	Halcion	alpha hydroxytriazolam 4-hyroxytriazolam 3-HM*
4.	Lorazepam	Ativan	lorazepam
5.	Chlorazepate	Tranxene	nordiazepam oxazepam
6.	Flurazepam	Dalmane	OH-ethylflurazepam desalkylflurazepam
7.	Temazepam	Restoril	3-OH diazepam oxazepam
8.	Oxazepam	Serax	oxazepam
9.	Chlordiazepoxide	Librium	demoxepam nordiazepam norchlordiazepoxide oxazepam
10.	Prazepam	Centrax	nordiazepam oxazepam

* Hydroxylated benzophenone metabolites of alprazolam and triazolam.

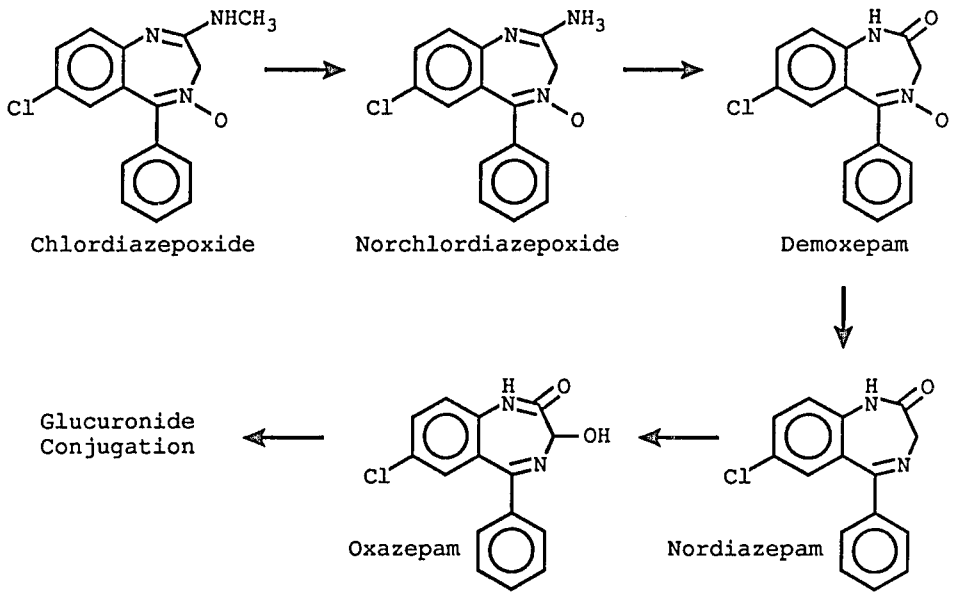

Figure 1a. Metabolism of Chlordiazepoxide

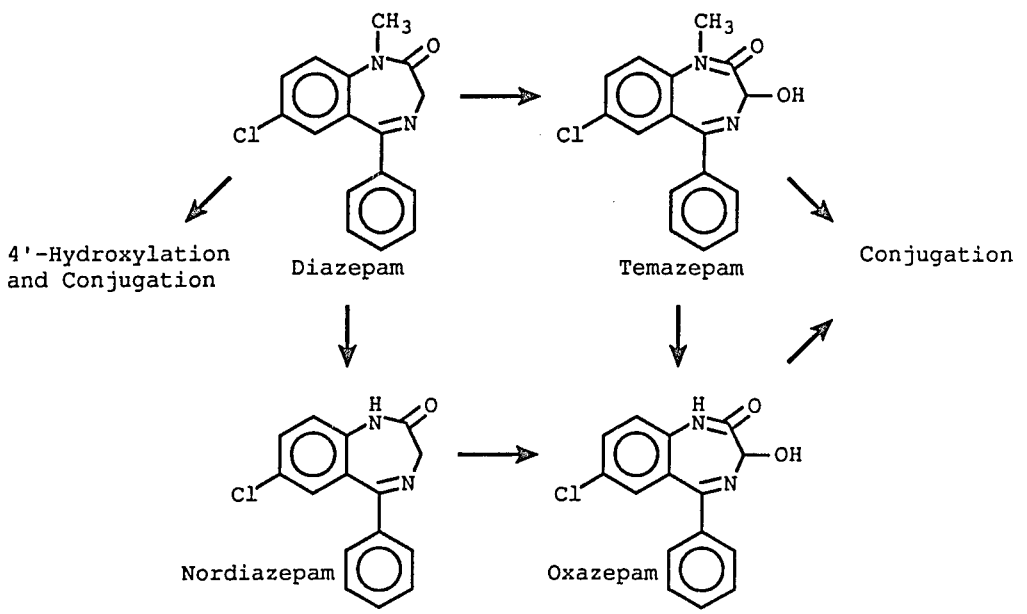

Figure 1b. Metabolism of Diazepam

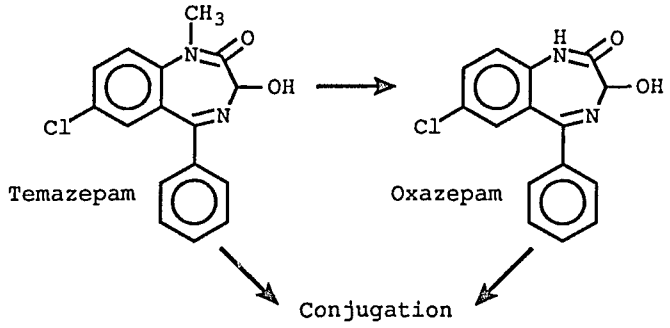

Figure 2a. Metabolism of Temazepam

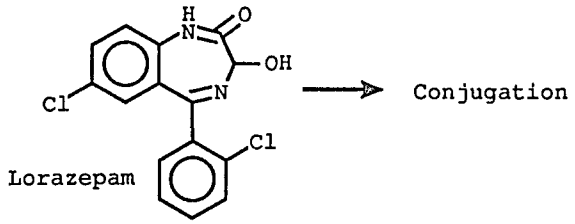

Figure 2b. Metabolism of Lorazepam

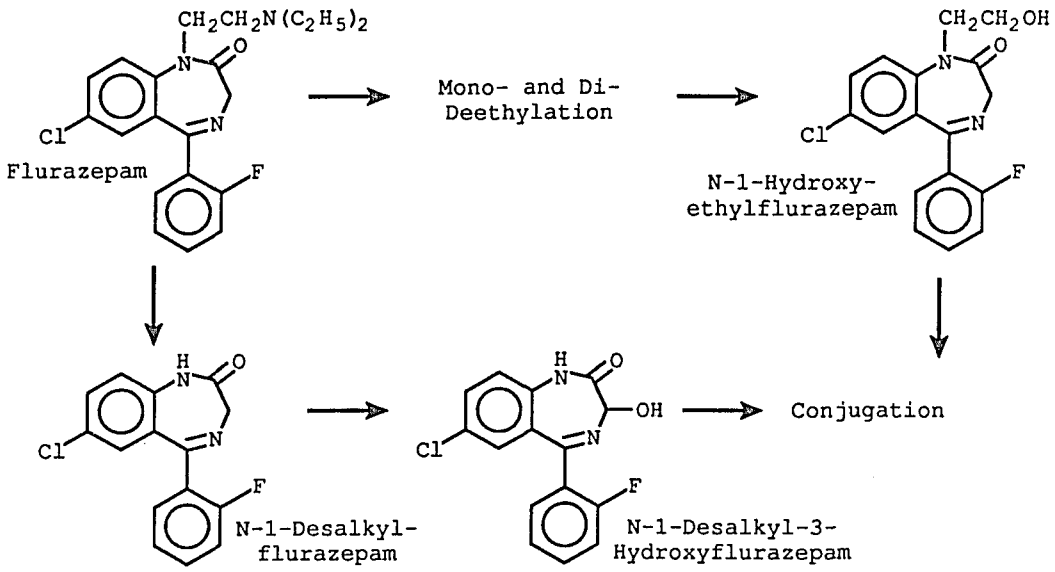

Figure 2c. Metabolism of Flurazepam

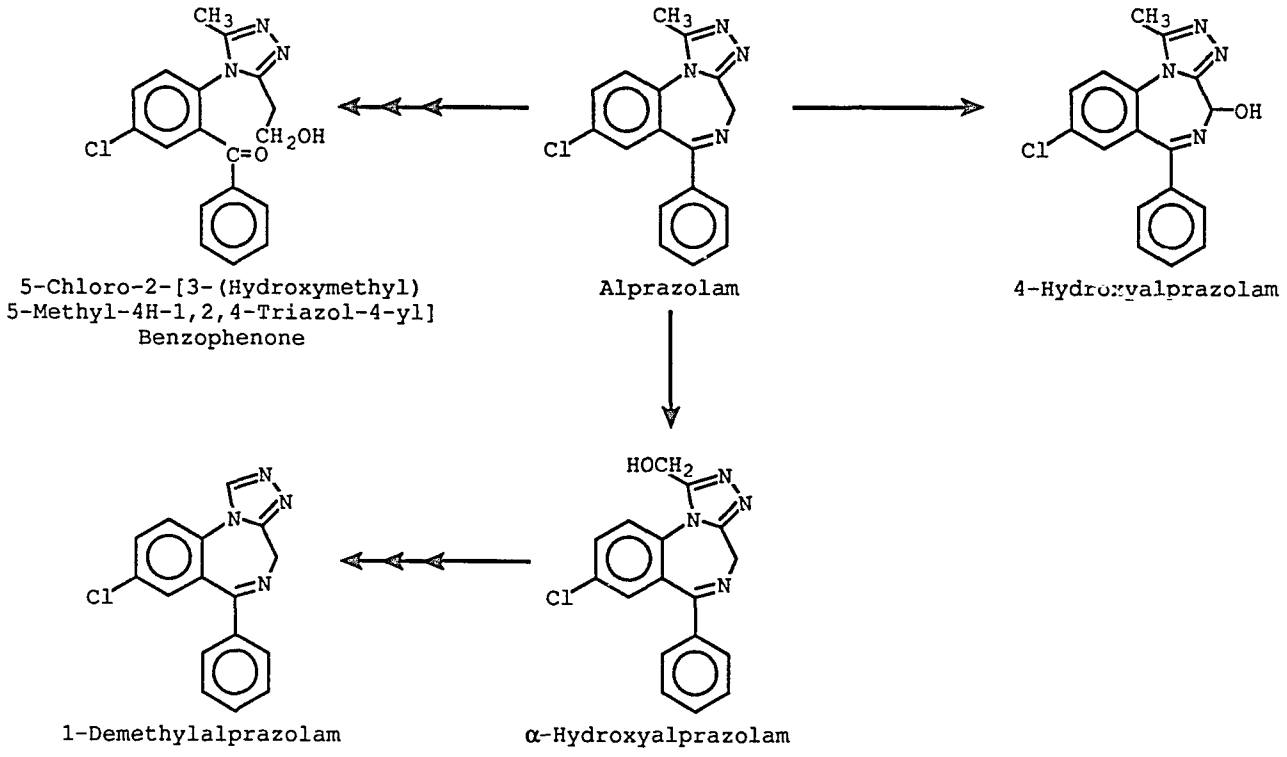

Figure 3. Metabolism of Alprazolam

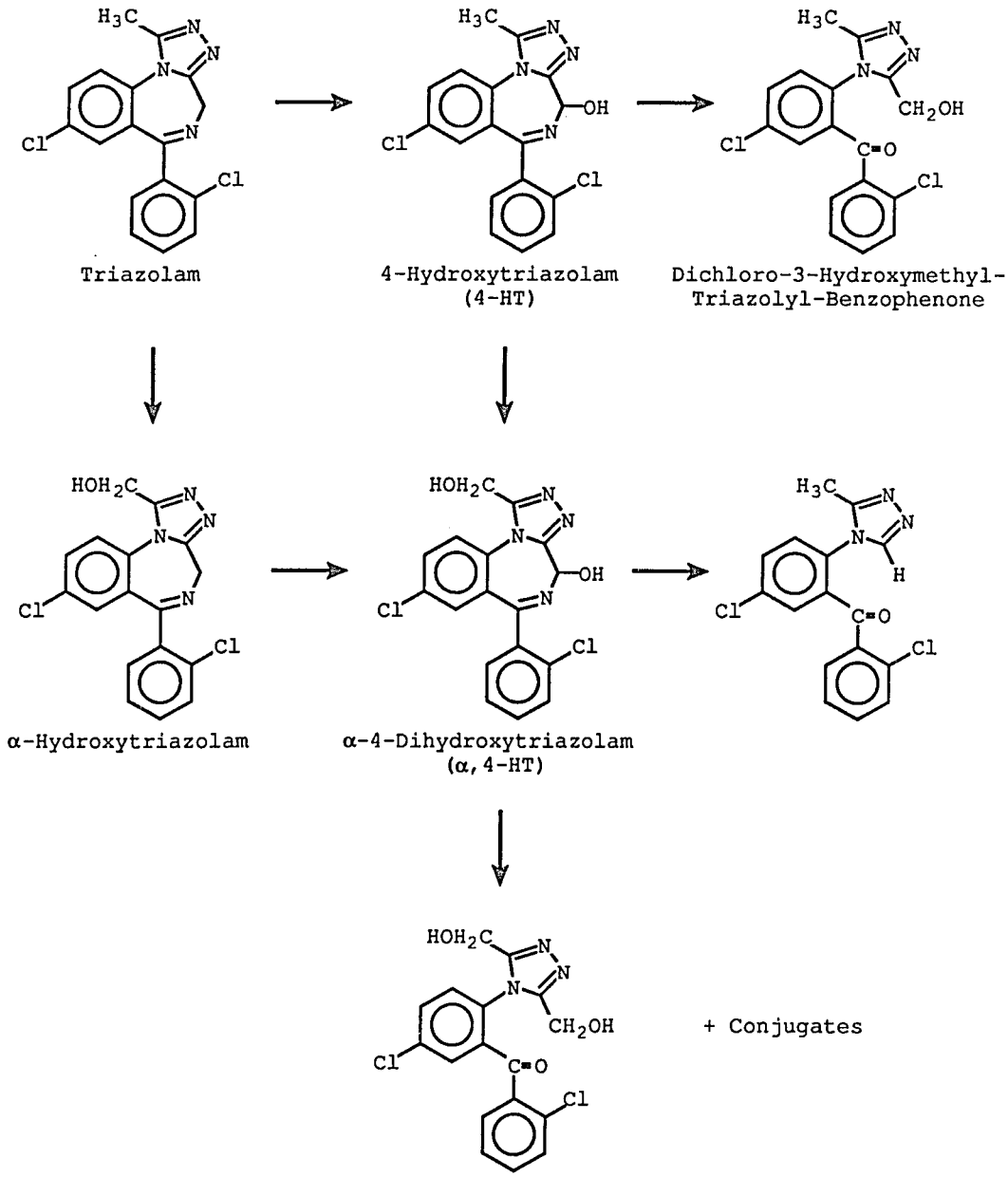

Figure 4. Metabolism of Triazolam

MATERIALS AND METHODS

Materials

Equipment:

A Hewlett Packard (Avondale, PA) benchtop GC/MS system was used for the development of the GC/MS data. This system consists of the following components all of Hewlett Packard manufacture; a series 200 computer with color monitor and 20 MB winchester disc drive, HP 5890 gas chromatograph configured for capillary chromatography, HP 9890 autoinjector, HP 5790 MSD (quadrupole, mass selective detector) with a capillary direct interface, and a printer. The MSD is configured for electron ionization using a 70 mev potential. The computer was equipped with revision 3.1.1 software.

Four capillary columns with the following specifications were used for the acquisition of GC/MS data:

1) Hewlett Packard "Ultra 1," methylsilicone stationary phase. Length, 12 meters. Inside diameter, 0.2 mm. Film thickness, 0.33 micron.
2) Hewlett Packard "Ultra 2," 5% phenyl methylsilicone stationary phase. Length, 12 meters. Inside diameter, 0.2 mm. Film thickness, 0.33 micron.
3) J & W Scientific (Folsom, CA) "DB-17," 50% phenyl methylsilicone stationary phase. Length, 10 meters. Inside diameter, 0.18 mm. Film thickness, 0.3 micron.
4) J & W Scientific "DB-1," methylsilicone stationary phase. Length, 20 meters. Inside diameter, 0.18 mm. Film thickness, 0.4 micron.

Gas chromatography was performed using a model 5790 Hewlett Packard gas chromatograph. This instrument was equipped with a capillary on column injection port, nitrogen phosphorus (N/P) detector and a J & W Scientific "DB-17" capillary column. Specifications for this column are as follows: Length, 15 meters. Inside diameter, 0.32 mm. Film thickness, 0.25 micron.

Equipment used for radioimmunassay include the following:

1) TECAN (Hillsborough, NC) model 505 robotic sample processor.
2) ICN Micromedic (Horsham, PA) model 4/200 gamma counter.
3) Beckman (Palo Alto, CA) model J6B centrifuge.

Reagents:

1) Pentafluoropropionic Anhydride (Pierce Chemical Co.)
2) Trifluoroacetic Anhydride (Pierce Chemical Co.)
3) Acetic Anhydride (E.M. Science)
4) MSTFA (N-Methyl-N-Trimethylsilyltrifluoroacetamide) (Pierce Chemical Co.)
5) MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide). (Pierce Chemical Co.)
6) Dichlorodimethylsilane (Pierce Chemical Co.)
7) Hydrochloric Acid, concentrated (Mallinckrodt)
8) Ammonium Hydroxide (Mallinckrodt)
9) Hexane, reagent grade (Mallinckrodt)
10) Toluene, reagent grade (Mallinckrodt)
11) Isopropanol, reagent grade (Mallinckrodt)
12) Potassium Hydroxide, 10N. Prepared by dissolving 140 grams potassium hydroxide pellets (Mallinckrodt) in dionized water to

a final volume of 250 ml.

13) 1.1 N Acetate buffer, pH 5.2. Prepared as follows: Dissolve 14.95 grams sodium acetate (Mallinckrodt) and 2.2 ml glacial acetic acid (Mallinckrodt) to a final volume of 100 ml in deionized water.

14) Abuscreen Radioimmunoassay for Benzodiazepines. Product No. 43343 (Roche Diagnostic Systems, Nutley New Jersey)

Drugs and Drug Metabolites:

1) Oxazepam-D5, 100 mg% methanolic solution. Prepared by dissolving in methanol, 5 milligrams oxazepam-D5 (Sigma Chemical Co.) to a final volume of five ml.

2) Nordiazepam-D5, 100 mg% methanolic solution. Prepared by dissolving in methanol, 5 milligrams nordiazepam-D5 (Sigma Chemical Co.) to a final volume of five ml.

3) Temazepam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg temazepam (Sigma Chemical Co.) to a final volume of 50 ml.

4) Nordiazepam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg nordiazepam (Sigma Chemical Co.) to a final volume of 50 ml.

5) Oxazepam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg oxazepam (Sigma Chemical Co.) to a final volume of 50 ml.

6) Lorazepam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg lorazepam (Wyeth-Ayerst) to a final volume of 50 ml.

7) HO-ethylflurazepam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg HO-ethylflurazepam (Hoffmann-LaRoche) to a final volume of 50 ml.
8) Alpha hydroxyalprazolam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg of alpha hydroxyalprazolam (Upjohn) to a final volume of 50 ml.
9) Alpha hydroxytriazolam, 100 mg% methanolic solution. Prepared by dissolving in methanol, 50 mg of alpha hydroxytriazolam (Upjohn) to a final volume of 50 ml.

Preparation and GC/MS Analysis of Benzophenone Derivatives

In developing a new GC/MS assay, the first task was to choose an appropriate extraction and derivatization procedure from the many found in the literature. Based partially on the volume of literature devoted to benzophenones, it was decided to look first at the feasability of this derivative. The method described by Verebey et al.(1982) seemed particularly well suited for GC/MS analysis and was used as a starting point for the development of the new assay.

Benzophenones were prepared as follows. Individually, 10 µl of oxazepam-D5, oxazepam, temazepam, HO-ethylflurazepam and lorazepam were added to 16x125 mm screw-top tubes containing 2 ml deionized water using the 100 mg% methanolic standards of the respective compounds. This resulted in a concentration of 5 µg/ml. To these tubes was then added 1.0 ml conc. HCl, the tubes were capped and placed in a 100 deg. C. hot block for one hour. Following hydrolysis, the tubes were allowed to cool and 1.0 ml 10N KOH followed by 0.4 ml conc. ammonium hydroxide

was added. Next, the benzophenones formed were extracted with 5 ml hexane, the extract transferred to a new 16x100 mm tube and evaporated to dryness. Extracts for each of the benzophenone derivatives were reconstituted in 200 µl toluene and analyzed using full scan GC/MS to evaluate the chromatographic performance of each benzophenone. The HP "Ultra 2," 5%, phenyl methylsilicone column was used for this purpose. Injections were splitless and the oven temperature was programmed from 100 to 320 deg. C. The MS acquired ions in the range of 40 to 550 amu. Full scan spectra for each compound were compared to published spectra to verify identity.

It became obvious that it would be necessary to improve the chromatographic performance of the benzophenones in order to quantitate in the low ng/ml range. Several papers describe the acylation of benzophenones with a variety of agents for this purpose. Pentafluoro-propionic anhydride was tried first. The procedure used to form the benzophenones was repeated except that the extracts were evaporated in 13x100 mm screw-top tubes to allow derivatization of the benzophenones. To the benzophenone extract contained in the 13x100 was added 100 µl hexane and 100 µl pentafluoropropionic anhydride. The vials were sealed and placed in a 70 deg. C. hot block for 30 minutes. The vials were then allowed to cool and the derivatization mixture was evaporated with low heat and air. The derivatized benzophenones were reconstituted with toluene and then chromatographed on the GC/MS. The full scan mass spectra for each were collected. The chromatography of the benzophenone-PFP derivatives were compared on three columns of differing polarity. These were the 12 meter, 5% phenyl methylsilicone column, the

12 meter, methylsilicone column and the 10 meter, 50% phenyl methylsilicone column.

Alpha hydroxytriazolam (alpha-HT) and alpha hydroxyalprazolam (alpha-HA) do not form benzophenones but can be acylated so it was hoped that the analysis of these compounds could be incorporated into a common extraction and derivatization method. Ten micrograms of alpha-HT and alpha-HA in 5 ml reaction vial were reacted with pentafluoro-propionic anhydride using the procedure described for the benzophenones. The derivatives were injected on the MS in SCAN to evaluate chromatography and collect the full scan spectra. Acyl derivatives of both compounds were prepared using acetic anhydride as follows. To ten micrograms of alpha-HA and alpha-HT contained in a 5 ml reaction vial were added 100 µl ethylacetate and 3 drops acetic anhydride. The vials were sealed and placed in a 70 deg. C. hot block for 30 minutes. Following derivatization, the reaction mixture was evaporated, reconstituted with 400 µl toluene and 2 µl injected on the GC/MS. The derivatives of alpha-HA and alpha-HT were chromatographed on the GC/MS using four different columns. These were the 12 meter, 5% phenyl methylsilicone column, the 12 meter, 0.2 mm I.D methylsilicone column the 20 meter methylsilicone column and the 10 meter, 50% phenyl methyl silicone column. The same derivative was also injected on a HP5790 capillary GC. This instrument was equipped with an on column injection port and a 15 meter, 50% phenyl methylsilicone capillary and N/P detector. The instrument was programmed from 100 to 320 deg. C. Pentaflouro derivatives for alpha-HA and alpha-HT were also injected on this instrument using the same conditions.

Preliminary Development of a Benzophenone-PFP GC/MS Assay

The GC/MS was programmed for SIM analysis using three characteristic ions selected from the full scan spectra for each of the analytes. Two ions were used for the acquisition of the internal standard. The ions (m/z) monitored for the benzophenone-PFP derivatives were, ACB-D5-PFP, m/z 382, 263; ACB-PFP, m/z 377, 258, 260; MACB, m/z 286, 356, 338; lorazepam benzophenone-PFP, m/z 411, 292, 300; HO-ethyl-flurazepam-PFP, m/z 274, 422, 402. A macro program was written and a calibration table prepared to automate the instrument for quantitative analysis. A mixture of the benzophenone-PFP derivatives was prepared and injected on the instrument to test the instrument settings.

Standard solutions in urine were prepared using benzodiazepine negative human urines provided by Poisonlab Inc., a NIDA certified forensic laboratory. These specimens had been previously tested for benzodiazepines and found to be negative by Roche RIA using a 100 ng/ml limit of analysis. Specimens that test negative are normally discarded after analysis and therefore represent an ideal source of blank urine. Urine calibration standards at concentrations of 50, 100, 200, 500, 1000 and 2000 ng/ml were prepared using the following procedure. Certified negative urine was pooled and used to partially fill volumetric flasks, one for each of the standards. Two hundred fifty ml flasks were used for the preparation of the 50 and 100 ng/ml calibrators and one hundred ml flasks for all others. To each was spiked oxazepam, temazepam, lorazepam and HO-ethylflurazepam using the 100 mg% methanolic standards sufficient to achieve the indicated concentrations. These amounts were

12.5, 25, 20, 50, 100 and 200 µl. The flasks were filled to the line, mixed and 2 ml aliquotted into 16x125 screw-top tubes.

The following extraction and derivatization procedure was applied to the urine standards.

1) To 2 ml urine in a 16x125 mm screw top tube, add 20 µl of a 1 mg% oxazepam-D5 internal standard. This results in a final internal standard concentration of 100 ng/ml.
2) To each tube add 1.0 ml concentrated HCl, seal with a teflon lined screw-top cap and place tubes in a 100 deg. C. hot block for 30 minutes
3) Remove tubes from hot block and allow to cool. Add 1.0 ml of 10N KOH and allow to cool.
4) To all tubes add 0.5 ml conc. ammonium hydroxide and 5 ml hexane. Extract on reciprocating shaker for 20 minutes. Centrifuge and remove organic layer to 13x100 screw-top tubes.
5) Evaporate hexane with low heat (approx 50 deg. C.) and passing air over the surface. Allow vials to cool.
6) To each tube add 200 µl hexane and 200 µl pentafluoropropionic anhydride. Cap tubes and place in 70 deg. C. hot block for 30 minutes.
7) Allow vials to cool, and evaporate to dryness without heat. Reconstitute with 200 µl toluene and transfer to conical injection vials.

Extracts of the urine calibrating standards prepared in this manner were injected on the MS using the new quantitative method to test

linearity. For this and subsequent work with the benzophenone-PFP derivatives, the 10 meter, 50% phenyl methylsilicone column was used. A total of 15, 100 ng/ml standards were similarly extracted and tested. The data from these injections were used to determine the within run precision of the assay at the 100 ng/ml level.

Preliminary Development of Benzodiazepine-TMS and TBDMS GC/MS Assays

Experiments were undertaken to determine the feasibility of analyzing benzodiazepines intact as their TMS or TBDMS derivatives. The TMS derivatives of oxazepam, nordiazepam, temazepam, HO-ethylflurazepam, lorazepam, alpha-HT, alpha-HA, oxazepam-D5 and nordiazepam-D5 were prepared as follows. Five µl of the 100 mg% methanolic standard for each drug was evaporated in separate 13x100 mm screw top tubes. To each vial was added 200 µl ethyl acetate and 100 µl MSTFA. The tubes were then sealed and derivatized in a 70 deg. C. hot block for 30 minutes. Following derivatization, the vials were allowed to cool, and the contents were transferred to conical injection vials. Two microliters were injected on the GC/MS to acquire full scan mass spectra for each of the compounds. This data was acquired using the 12 meter methylsilicone column. The instrument was temperature programmed from 100 to 320 and the MS scanned the range 40-550 amu. TBDMS derivatives for the same compounds were prepared in the same way except that MTBSTFA was substituted for MSTFA. The full scan spectra were also acquired.

Using the initial chromatographic results obtained by the injection of the individual TMS and TBDMS derivatives, an oven temperature profile was selected and entered into new method files for both sets of

derivatives. Two ions for the internal standards and three ions for each analyte were selected from the full scan spectra and entered into method files for SIM acquisition. The ions monitored (m/z) for the TMS substituted benzodiazepines were, nordiazepam, m/z 341, 327, 342; oxazepam-D5, m/z 435, 433; oxazepam, m/z 429, 430, 431; temazepam, m/z 343, 357, 372; lorazepam, m/z 429, 431, 430; HO-ethylflurazepam, m/z 288, 360, 289; alpha-hydroxyalprazolam, m/z 381, 383, 396. Ions monitored for the TBDMS derivatives are listed in Table 7. Macro programs were written and calibration tables prepared to automate the instrument for quantitative analysis. To test the instrument settings for each of the two newly created GC/MS methods, mixtures of TMS and TBDMS derivatives of the combined benzodiazepines were prepared and injected using the new methods.

To cleave glucuronide conjugates from the urinary metabolites, glucuronidase was tested as an alternative to acid hydrolysis because heating the conjugates in acid would form the benzophenones. Sigma product No. G-0876 containing approximately 120,000 units/ml B-glucuronidase was purchased for this purpose. The activity of the enzyme was measured using a procedure included with the product. The assay involves incubation of the diluted enzyme at 37 deg. C. and at a pH of 5.0 using phenolphthalein glucuronide as the substrate. Hydrolysis of phenolphthalein glucuronide liberates phenolphthalein resulting in an increased absorbance at 540 nm. which is recorded on spectrophotometer (Gilford model S3). The enzyme activity is determined by comparison with a standard curve. One unit of glucuronidase activity by definition will liberate 1.0 µg of phenolphthalein from

phenolphthalein glucuronide per hour at pH 5.0 at 37 deg. C. Using the manufacturer's procedure, the activity of the enzyme was measured at 140,000 units/ml. Published procedures generally use approximately 2000 units enzyme per ml urine buffered to pH five.

Extraction of the benzodiazepines and their unconjugated metabolites was accomplished using a simple liquid/liquid extraction procedure. The steps used in the hydrolysis, extraction and derivatization of samples prior to GC/MS analysis are as follows;

1) To two ml urine in a 16x125 mm screw-top tube add 20 µl internal standard (2 mg% oxazepam-D5/nordiazepam-D5).
2) Add 0.5 ml of 1.1M, pH 5.0 acetate buffer and 50 µl (approximately 3500 units/ml) B-glucuronidase (Sigma G0876). Incubate 4 hours at 50 deg. C. or overnight at room temperature.
3) Add 3 drops concentrated ammonium hydroxide and five ml t-butylether/toluene/isopropanol (75:24:1). Cap tubes and mix or shake for 30 minutes.
4) Centrifuge and remove organic layer with a disposable transfer pipette into a 13x100 mm screw-top tube.
5) Evaporate to dryness, then add 200 µl ethylacetate and 100 µl MSTFA or MTBSTFA. Cap tubes and place in a 70 deg. C. hot block for 30 minutes (100 deg. C. hot block for TBDMS derivative).
6) Allow tubes to cool, then transfer contents to conical injection vials. Cap vials and inject 2 µl on GC/MS.

Linearity and precision of these two methods were evaluated in a manner analogous to that used to evaluate the benzophenone method. A series of standard solutions were prepared using blank urine containing all the drugs of interest at concentrations of 50, 100, 200, 500, 1000 and 2000 ng/ml. The procedure used to prepare these standards is identical to the one used in the preparation of the benzophenone standard solutions, except that two additional drugs were added, nordiazepam and alpha-HA. These were extracted using the procedure described and derivatized with either MSTFA or MTBSTFA and injected on the GC/MS. Concentration vs. response curves were generated from this data to assess linearity. Next, a series of standards spiked to 100 ng/ml were similarly prepared, extracted and tested. This data was acquired using the 12 meter methylsilicone column.

Validation of a Benzodiazepine-TBDMS GC/MS Assay

The initial work done with the three series of derivatives indicated that a method based on the TBDMS derivatives would be most suitable for a rigorous workup. Validation of the final procedure involved testing the method to determine: (a) the limits of detection and limits of quantitation (b) ion ratio stability vs. concentration (c) potential interfering substances (drugs) (d) extraction efficiency (e) percent contribution by the internal standards to the D 0 ion, and (f) accuracy. Linearity and within run precision at a tentative limit of analysis of 100 ng/ml was determined in the preliminary method workup. Finally, a confirmation limit of analysis would be selected based on the analysis of a large population of RIA positive urines.

This study, performed over a period of weeks, would also serve to establish the day to day precision by the analysis of controls, spiked at various concentrations, included with each run. This final phase of the validation is found in the succeeding section "RIA and GC/MS Analysis of Forensic Urine Specimens."

For the purposes of this work, the definitions for the limit of detection (LOD) and limit of quantitation (LOQ) were taken from the NIDA Laboratory Inspector's Training Manual. These are as follows:

$$LOD = X + 3\ SD$$

$$LOQ = X + 10\ SD$$

X is the mean value for the concentration of the analyte in the negative urines tested. SD is the standard deviation of the concentrations of the analyte in the negative urines tested. The limit of detection and limit of quantitation are determined by the analysis 12 blank urines. The values obtained for these specimens are entered into the formulas described to arrive at the values for LOD and LOQ. These are representative of the noise contributed by both the matrix and the electronic noise of the instrument. The application of these formulas is similar to other techniques that define a limit of detection based on a signal to noise level. In addition to the use of this statistical approach to determine sensitivity, extracted standards at concentrations of 5, 10, and 25 ng/ml were also tested. This additional test is useful since the recommended NIDA computation does not take into account the stability of peak qualifier ratios at lower concentrations which for

GC/MS work is often the factor most likely to limit sensitivity.

Ion ratio stability over the linear range was determined as part of the earlier linearity study. This is expressed as the percentage change of each peak qualifier ratio over the stated linear range of the assay.

Because of the high specificity of GC/MS, the possibility of a structurally unrelated compound resulting in an incorrect identification is considered insignificant. The presence of various compounds including drugs in a urine may, however contribute to the ion current of one or more of the compounds of interest. If this occurs at the retention time of one of the analytes, it would prevent the identification and quantitation of the analyte, should it be present, since conditions for peak qualifier ratios would not be met. The sensitivity of the assay to potential interferences was determined by the analysis of a large specimen population. Those specimens for which interferences are detected were subjected to full scan GC/MS to identify the interfering compound.

Structurally related compounds, i.e., the benzodiazepines, are a more likely source of interference. Those compounds that were available and not already included in the assay were extracted and tested using the procedure described. These compounds included demoxepam, norchlordiazepoxide and desalkylflurazepam.

Extraction efficiency was determined by comparing the values obtained with extracted and nonextracted calibration standards. A mixed benzodiazepine calibration standard of 1500 ng/ml was prepared in methanol. Another standard at the same concentration was prepared in negative urine. The urine standard was extracted and derivatized using

the indicated procedure except that the methanolic 2 mg% oxazepam-D5/ nordiazepam-D5 standard was added after extraction. To 2 ml of the 1500 ng/ml methanolic standard was added the same amount (200 ng/ml) of the mixed internal standard. This was evaporated, derivatized and injected on the MS. Extraction efficiency was calculated by comparing the values obtained for the extracted standards with the values obtained for the nonextracted standards.

The deuterated internal standards used, namely oxazepam-D5 and nordiazepam-D5, represent a potential for interference by two means. First, if the material is not completely labeled with deuterium, the addition of the internal standard will result in the addition of the native drug as well. The second potential source of interference is in the spectra of the deuterated compounds. If these spectra contain significant abundances for ions also monitored for the native drug, quantitation and ion ratios will be adversely affected.

Both internal standards used have a stated enrichment of over 98% and should not represent a source of contamination. The test for enrichment is simply the calculation of the amount of native drug in blank specimens to which only the internal standards have been added. The contribution to the D 0 ions by the internal standard is predicted by examining the full scan spectra of the deuterated compounds for the presence of ions shared by the native drugs. If present, these ions are also observed in blank extracts.

To assure accuracy, generally accepted quality control procedures were used. The powdered form of the drugs were weighed into methanolic standards using a balance of certified accuracy. The full scan total

ion chromatograph (TIC) of these standards were examined for the presence of additional peaks that might indicate impurity. The purity of drug standards purchased or received from drug companies was usually indicated to be 98% or greater. Commercial controls containing oxazepam were tested to verify the accuracy of Q.C. materials prepared with this compound. Outside controls for the other compounds were not available.

RIA and GC/MS Analysis of Forensic Urine Specimens

Benzodiazepine positive human urine specimens were obtained from Poisonlab Inc. for reanalysis using the new TBDMS method. These specimens were received by Poisonlab for analysis from several sources including industrial clinics, private employers and from other drug testing laboratories. The majority of these specimens were submitted for pre-employment drug screens, however many specimens were received through work furlough or probationary programs. All positive specimens were frozen immediately after analysis at -20 deg. C. The testing protocol used at Poisonlab involves a the initial use of Roche radioimmunoassay followed by confirmation by GC/MS. The confirmation method used by this laboratory is the preliminary benzophenone-PFP method already described.

Most of the specimens obtained had been previously found positive for ACB, MACB or HO-ethylflurazepam. There was some difficulty in obtaining specimens containing alpha-HA since the confirmatory method in use at Poisonlab did not identify this metabolite and therefore many specimens that were likely to contain this metabolite were not retained. Only one specimen containing lorazepam could be obtained. Selection of

specimens was not random and therefore results from this small number of specimens may not reflect positive distributions in large specimen populations. The selection of specimens was designed mainly to challenge the capability of the assay by the analysis of specimens containing different combinations and concentrations of metabolites.

Benzodiazepine positive urine specimens were retrieved from frozen storage, thawed and aliquots transferred to 16x125 mm screw top tubes for reanalysis by both RIA and GC/MS. Roche RIA for benzodiazepines was performed on all aliquots following the manufacturer's instructions. RIA requires precise measurement of sample, tracer and antibody. These pipetting steps were performed using a TECAN RSP model 505 robotic sample processor. Specimens were evaluated as positive or negative based on a comparison to a 100 ng/ml oxazepam calibration standard tested with unknowns and provided with the kit. RIA counts of unknowns that are equal to or less than those of the standard were evaluated as positive. The linear range of the assay was determined by the analysis of urine standards containing oxazepam spiked to concentrations of 100, 200, 300, 400, 500, 600, 700 and 800 ng/ml. These were prepared in negative urine using the 100 mg% methanolic oxazepam standard in a manner similar to the procedure used for the preparation of the GC/MS urine standards.

GC/MS calibration standards and controls were prepared at concentrations consistent with NIDA requirements for GC/MS methods. These require as a minimum a standard solution at the limit of analysis and at 50% below the cutoff. Two open controls, one positive and one negative, must also be included in each run. Open controls are those controls

that can be identified by the analyst. For the analysis of case specimens, two blind controls must also be included in each analytical run. These are controls that appear to the analyst as normal specimens. Controls are prepared in the same manner as urine standards except that they are prepared using different lots of methanolic standards. The values obtained for these controls are used to validate the analytical run for accuracy and the method over time for precision. Only open controls were used in the validation work.

Calibration standards were prepared at concentrations of 50, 100 and 1500 ng/ml. Controls were prepared at concentrations of 0, 100 and 1000 ng/ml. RIA positive aliquots were extracted for GC/MS analysis in groups of 15 or less along with the calibration standards and controls, using the protocol described. A total of 66 specimens were tested in this manner in 6 separate runs. Prior to analysis, normal maintenance was performed on the GC/MS including tuning and replacement of the injection port liner and septum.

Tuning of the MS is performed daily as recommended by Hewlett Packard using the "Autotune" program supplied as part of the software. Using perfluorotributylamine (PFTBA) as a reference standard, the program adjusts lense voltages, electron multiplier settings and other electronic parameters in such a way that a normalized spectrum for the reference standard is produced. These new tune parameters are then stored to a tune file which can be included as part of a method file. A permanent hardcopy of these tune parameters is generated after each autotune which serves to document instrument conditions at the time of analysis. Daily autotunes are also useful in the diagnosis of a variety

of common problems such as a dirty source, vacuum leaks, carrier gas contamination and a host of other problems.

Prior to analysis, the glass injection port liner was replaced with a clean, silane treated liner. The procedure for the preparation of the liner is as follows:

1) Clean the used liner with a fine wire brush to remove carbon deposites, rinse with acetone and allow to dry.
2) Place liner in a 16x125 mm screw-top tube and add 15 ml dichlorodimethylsilane (10% in toluene).
3) Cap tube and allow to stand for 20 minutes.
4) Remove the dichlorodimethylsilane to waste and rinse the liner four times with toluene. Rinse the liner four times with methanol and allow to dry.
5) Insert a fine plug of silanized glass wool into the liner, 1 cm from the end.

The three extracted urine calibration standards were injected on the GC/MS and a three point calibration curve was generated. Ion ratios and retention times of the 100 ng/ml standard were used to calibrate the instrument since this is the tentative limit of analysis. After this calibration procedure had been performed, the controls and specimens were injected.

RESULTS

Preparation and GC/MS Analysis of Benzophenone Derivatives

Using the modified method of Verebey, et al. (1982) the benzophenones 2-amino-5-chlorobenzophenone (ACB) and 2-methylamino--5-chloro-benzophenone (MACB) were generated, extracted and injected on the GC/MS using full scan acquisition. The identity of these compounds were confirmed by comparing the mass spectra acquired against spectra published by Pfleger, et al. (1985). The procedure was also found to produce the benzophenone derivatives of HO-ethylflurazepam and lorazepam, again confirmed by comparison of the spectra generated against published spectra. ACB, MACB and the lorazepam benzophenone chromatographed reasonably well and showed symmetrical peaks. HO-ethylflurazepam benzophenone chromatographed poorly as a broad, asymmetrical peak. The reaction of these benzophenones with pentafluoropropionic anhydride (PFPA) resulted in greatly improved chromatography for all four compounds. This was expected since a similar application had been reported with the same observed improvement in chromatography (Sanchez, et al. 1976).

The spectra of the pentafluoro derivatives were found to contain ions of high molecular weight. This is an advantage in MS analysis since interfering substances are less likely to produce ions in the high MW end of the spectrum. The identity of these derivatives were mostly confirmed by comparing the expected molecular weight to the observed molecular ions found in each of the spectra. Mass spectra for the benzophenone-PFP derivatives studied are illustrated in Figures 8a-e. The formation of PFP derivatives results in the addition of 146 amu to

the native compound. For the ACB, ACB-D5, MACB and the lorazepam-PFP derivatives, prominent molecular ions at the MW of the benzophenone plus 146 amu were found (Table 2). Further confirmation was facilitated by the presence of chlorine in all of these compounds. ACB, ACB-D5 and MACB contain one chlorine and the spectra for these compounds contained ions at M and M+2 with a relative abundance of the M+2 peak of approximately 33%. This was expected since the natural isotopic abundance of Cl 37 is 33% relative to Cl 35. Lorazepam contains two chlorines and as expected the spectra containes prominent ions at M+2 and M+4. The abundance of the M plus two ion, relative to the molecular ion was approximately 66%, consistent for molecules containing two chlorines.

The spectra for the PFP derivative of the flurazepam metabolite presented an interesting anomaly in that the apparent molecular ion is 23 amu higher than expected. A significant peak at the expected molecular weight was found, however. It is difficult to propose a structure for this compound given the discrepancy between the hypothetical molecular weight and the observed molecular ion. In any case, the identity of the material used was confirmed by comparison of the underivatized spectra to published spectra for this compound. Structures for the benzophenone-PFP derivatives are assumed to be analogous to the published structures for the acyl derivatives for these compounds.

Of the three columns tested with the benzophenone-PFP derivatives, the 10 meter, 50% phenyl methylsilicone, was found to be the most suitable. Chromatography using the 5% phenyl methylsilicone and

methylsilicone columns was reasonably good, except that resolution of ACB and MACB was incomplete, particularly at high analyte concentrations. This problem worsened with column degradation. Chromatography using the 50% phenyl methylsilicone column was consistently good and relatively less sensitive to column age or the concentrations of the compounds tested.

Derivatives of alpha-HA and alpha-HT could not be produced using pentafluoropropionic anhydride. Peaks were not observed when the reaction products of both compounds were chromatographed on the GC/MS using a methylsilicone column programmed to a temperature of 325 deg. C. The same reaction products were subjected to gas chromatography using a HP 5790 GC, also programmed to it's temperature maximum. Again, peaks were not observed.

Since PFPA was found not to react with either alpha-HA or alpha-HT, acyl derivatives were investigated as a means of incorporating these metabolites into a comprehensive assay. Only poor chromatography was observed when these derivatives were injected on the GC/MS. Attempts to improve chromatography by the use of various columns differing in length and polarity were largely unsuccessful. The best results were obtained using the 12 meter, methylsilicone column temperature programmed to its temperature maximum of 325 deg. C. Using these conditions the acyl derivative of alpha-HA could be chromatographed but only as a broad, asymmetrical peak. The full scan spectrum of this peak contained the molecular ion expected for the acyl derivative of alpha-HA. Alpha-HT, which has a higher retention index, was not chromatographed under these conditions.

Good chromatography for the acyl derivatives of both compounds was obtained using a HP 5790 gas chromatograph, equipped with a 50% phenyl methylsilicone capillary column. Both compounds eluted as symmetrical peaks but with long retention times, requiring an oven temperature of approximately 300 deg. C. The published retention index for the acyl derivative of alpha-HT is 3200 (Pfleger, et al. 1985). This very high retention index is consistent with these findings.

The inability to form acyl derivatives of the triazolam and alprazolam metabolites that would chromatograph in the GC/MS system, represented a major problem. It meant that these important metabolites could not be easily incorporated into the benzophenone method. These observations were unexpected since good GC chromatography could be obtained. It appeared that the GC/MS system was inferior in terms of chromatographic performance when compared to a comparable GC system particularly for compounds with high retention indices.

Preliminary Development of a Benzophenone-PFP GC/MS Assay

Analysis of a series of urine calibrators over the range of 50 to 2000 ng/ml showed a linear relationship for the benzophenones tested, namely, ACB, MACB, lorazepam and HO-ethylflurazepam. The correlation coefficients, used as a measure of linearity, exceeded 0.995 for all four compounds. Although the efficiency of the extraction was not measured, sensitivity was adequate to detect the benzophenone derivatives at concentrations as low as 50 ng/ml using a 2 ml extract. Chromatographs were characterized by symmetrical peaks and were interference free. All compounds eluted well below the 300 deg. C.

temperature maximum of the 50% phenyl methylsilicone column used.

Within run precision, expressed as the coefficient of variation, for the analysis of 15, 100 ng/ml standards was found to be as follows: ACB = 3%, MACB = 8%, lorazepam = 8%, HO-ethylflurazepam = 12%. As expected, the C.V. for ACB was lowest since oxazepam-D5, used as the internal standard, is converted to ACB-D5 and therefore represents the best internal standard for this compound. NIDA testing requires the ability to quantitate to within 20% of the known value. For a method to consistently produce this level of accuracy and precision, a new method should deliver a within run coefficient of variation on the order of 5%. Although this level of precision was obtained for ACB only, the other compounds were not far from the 5% target, suggesting that further development work might yield the desired precision.

Preliminary Development of Benzodiazepine-TMS and TBDMS Assays

TMS derivatives were investigated because they would identify the actual drug metabolite instead of a hydrolysis product. MSTFA was found to react with all the benzodiazepines tested to form TMS derivatives. The full scan spectra for each are illustrated in Figures 9a-i. Unlike the acyl derivatives of alpha-HA and alpha-HT, the TMS derivatives of these compounds exhibited reasonably good chromatographic properties. Both eluted at temperatures approaching the temperature maximum of the column, but peak shapes were Gaussian, an indication that quantitative precision might be attainable since good chromatography is consistent with good precision. Peak shapes for the other derivatives were also good.

The theoretical molecular weights of the derivatives were consistent with the molecular ions observed in the spectra. The addition of one TMS group results in a derivative with a mass of 72 amu above the underivatized compound. Two of the compounds, lorazepam and oxazepam, were substituted by two TMS groups as indicated by the presence of the molecular ion with a mass 144 greater than the mass of the underivatized metabolite (Table 3). It is assumed that these compounds were substituted at the N 1 nitrogen and the 3 hydroxyl positions.

Mass spectra of TMS derivatives are generally characterized by a prominent ion at M-15. This corresponds to the loss of one methyl group from the trimethylsilyl group. Compounds substituted by two TMS groups often generate spectra with ions at both M-15 and M-30 corresponding to the loss of methyl groups at one or both TMS groups. All of the TMS spectra contained prominent ions at M-15 or M-30. The spectra for these compounds also contained ions at M+2, which is expected of chlorine containing compounds. For most of the benzodiazepine-TMS derivatives, the molecular ion was found to have a high relative abundance.

Initial results for extracted urine standards indicated that the method was capable of quantitation in the low ng/ml range with linearity to approximately 2000 ng/ml. Chromatography was erratic however, characterized by large intra-injection changes in the abundances of the oxazepam-D5 internal standard. There appeared to be more baseline noise than normal and peak shapes were less symmetrical than expected from the evaluation of the non-extracted drugs. At the lower concentrations used in the linearity study, the peak shape for alpha-HT was poor, and as a result, no further attempts were made to include this compound in later

work. Concentration vs. response curves (linearities) for all compounds were best described by a straight line with correlation coefficients exceeding 0.995. Precision for the drugs tested as measured by the within run coefficient of variation were as follows: nordiazepam 12%, oxazepam 2%, lorazepam 5%, 3-OH diazepam 9%, HO-ethylflurazepam 12% and alpha-HA 20%.

A common problem with TMS derivatives is their sensitivity to moisture resulting in instability. It is suspected that the variation in abundances for the internal standard and other TMS compounds were due to the inherent chemical instability of these compounds.

TBDMS derivatives were investigated because they have been reported to be approximately 10,000 times more stable to hydrolysis for some compounds than the corresponding TMS derivatives. The derivatization reagent adds a tert-butyl-dimethylsilyl group to hydroxyl and amino groups on the molecule. The base peak for these derivatives is generally found at M-57, which corresponds to the loss of a tert-butyl fragment from the molecule (Table 4). This rule held for all the benzodiazepines tested including those substituted at two positions. The molecular ion was identified for all the compounds tested, but unlike the TMS derivatives, the abundance of the molecular ion was relatively low. Spectra for the TBDMS derivatives are illustrated in Figures 10a-i.

Chromatography for most of the compounds tested was good, although retention times for the TBDMS derivatives were longer than for the corresponding TMS derivatives. This was expected since these compounds have higher molecular weights. The problem was offset by using an oven

temperature program with higher initial and final temperatures. Alpha-HA eluted at the temperature maximum of the column as a symmetrical peak. Alpha-HT chromatographed poorly and eluted at very long retention times.

Extracted urine calibration standards gave a linear response for all compounds using the same extraction technique used to evaluate the TMS derivatives. The linear range for all compounds except nordiazepam and HO-ethylflurazepam was found to be at least 50-2000 ng/ml. Column overload was found to limit the linearity for nordiazepam and HO-ethylflurazepam to 1500 and 1000 ng/ml respectively. By lowering the injection volume, linearity for these compounds could be extended to at least 2000 ng/ml. Correlation coefficients for all compounds exceeded 0.995. Within run precision for all compounds was below 6%. These data together with subjective impressions of assay performance indicated that the TBDMS derivative would be the best candidate for a rigorous method workup.

Validation of a Benzodiazepine-TBDMS GC/MS Assay

Specifications for the TBDMS method are listed in Tables 5 and 6. The limits of detection and limits of quantitation were found to be on the order of 0.03-4 ng/ml, calculated using the NIDA recommended protocol. This method of computation represents a source of controversy, since in general, the results obtained are unrealistically low for most GC/MS assays. These formulas seem to be used by NIDA primarily in the intralaboratory comparison of assay "sensitivity". These values should therefore not be used without examining other criteria. For

example, the computed LOQ for lorazepam is 0.5 ng/ml. Five ng/ml was barely detectable in a sample prepared to contain this amount.

The analysis of extracted urine standards at 5, 10 and 25 ng/ml was performed as an independent measure of sensitivity. All benzodiazepines contained in the 5 ng/ml standard were identified with the exception of nordiazepam which was correctly identified at the 10 ng/ml level. The criteria used here for detection or identification are the same criteria used for the identification of these analytes in unknown specimens. This involves the comparision of peak qualifier ratios and retention times of the sample to those of the tentative limit of analysis calibration standard. Retention times of the analytes must be within 2% and the peak qualifier ratios must be within 20% of the corresponding ratios of the extracted urine 100 ng/ml calibration standard. Three ions are acquired for all analytes, one of which is used for quantitation. The remaining two are used for identification and are referred to as peak qualifiers. The abundance ratio of the qualifying ion to the quantitative ion is the peak qualifier ratio. Since there are two qualifying ions, there are also two qualifier ratios per analyte. For example, lorazepam peak qualifiers are ions 513 and 493 with abundances relative to the quantitative ion 491 of 73% and 86% respectively. Identification of lorazepam in an unknown requires that all three ions be present at the correct retention time with peak qualifier ratios of 73% plus or minus 14.6% and 86% plus or minus 17.2%.

Enrichment for both the oxazepam-D5 and nordiazepam-D5 was found to exceed 99%. Blank urines to which only internal standard were added were tested and found to contain approximately 1 to 2 ng/ml of oxazepam

and nordiazepam resulting from the addition of the internal standards. Since 200 ng/ml internal standard is added to each tube, the purity, in terms or enrichment of these compounds, is greater than 99%. The spectra of the deuterated and nondeuterated standards were examined to verify that the ions acquired by the method were not shared. Both in terms of their spectra and enrichment, the deuterated internal standards were found not to be a source of interference.

Extraction efficiencies were as follows: nordiazepam 82%, oxazepam 86%, temazepam 83%, lorazepam 82%, HO-ethylflurazepam 77%, alpha-HA 66%. These data indicate that the choice of extraction solvent and the pH of the extraction are acceptable. Higher efficiencies could be obtained with multiple extractions, but this seems unnecessary. Additional extractions would complicate the analysis and would only be useful if the method lacked sensitivity. Efficiencies are sufficiently high that less polar solvents might be used in the extraction to yield cleaner extracts.

Peak qualifier ratios were found to be stable throughout the stated range of linearity. This was expected, since for many derivatives, the ions selected arise from isotopic variants of the same molecular fragment. An example are the ions 327, 328 and 329 acquired for nordiazepam. Table 7 lists all the ions acquired for this assay.

RIA and GC/MS Analysis of Forensic Urine Specimens

The RIA analysis of a series of oxazepam standards over the range of 100 to 800 ng/ml indicated that the Roche Abuscreen assay would not be useful for quantitative work above 200 ng/ml. Above this level, the

assay was not linear, as indicated by a flat calibration curve at these higher concentrations. Sensitivity for the Abuscreen is reported to be 5 ng/ml, meaning that it can reliably detect benzodiazepines at this level. It appeared that with this assay, linearity at higher concentrations was compromised in favor of sensitivity. A useful RIA and GC/MS correlation study using this kit and the GC/MS assay developed could not be performed since many RIA and GC/MS results were substantially above the linear range of the RIA assay. Specimen aliquots that were retested by this method and found to be positive at the recommended cutoff of 100 ng/ml were quantitated by GC/MS. A few of the specimens recovered from frozen storage tested somewhat below the 100 ng/ml cutoff and were rejected for further analysis.

The results of the GC/MS analysis of 66 specimens are listed in Table 8. The majority of the specimens tested in the range of 100 to 500 ng/ml for oxazepam, nordiazepam or temazepam. Eleven positives were found for HO-ethylflurazepam, three for alpha-HA and one for lorazepam. Three of the RIA positive specimens contained metabolites at concentrations below 100 ng/ml. Using a tentative limit of analysis of 100 ng/ml for the GC/MS assay, these would be evaluated as negative, representing 5% of the total specimens tested. In each of these three specimens, the total of the immunoreactive benzodiazepines was found to be greater than 100 ng/ml. All other specimens were found to contain at least one drug or metabolite at a concentration above 100 ng/ml.

An interference eluting at the retention time for the oxazepam-D5 internal standard was observed in one specimen found to contain HO-ethylflurazepam. Re-extraction and full scan GC/MS of the same

specimen found that the sample also contained propoxyphene and norpropoxyphene. Both compounds were then spiked into blank urine at a concentration of 5000 ng/ml and tested using the TBDMS method. Under these conditions, neither propoxyphene or norpropoxyphene were found to interfere. It is not known, therefore, what was responsible for the interference observed in this specimen. It was possible to successfully analyze this specimen by using nordiazepam-D5 as the internal standard to calculate the concentration of HO-ethylflurazepam. All other specimens were interference free. Some chromatographs did however contain unidentified peaks or noise. These did not interfere with the analysis but point the the need for cleaner extracts.

Norchlordiazepoxide and desalkylflurazepam did not interfere with the assay. Demoxepam, however did interfere with nordiazepam. This compound is nearly identical in structure to nordiazepam, and it appears that it may form the same derivative. This problem was not investigated further.

Between run precision for all compounds tested at 100 ng/ml (n = 6) was found to be below 10%. As expected, the best C.V. data was obtained for oxazepam and nordiazepam since their deuterated compounds were used as internal standards. A clean, silane treated liner was absolutely required to detect alpha-HA at the concentrations described. Quantitation of the other analytes were less sensitive to the condition of the liner. Alpha-HA could not be detected at all, even at high concentrations, if injected on a liner that had been previously used for other analysis.

Table 2. Structural Confirmation of PFP Substituted Benzophenones. Substitution of one PFP results in the addition of 146 amu to the parent compound.

COMPOUND	MOL. WT.	MOL. ION PFP DERIV.	OBSERVED MASS DIFFERENCE
ACB-D5	236	382	146
ACB	231	377	146
MACB	245	391	146
Lorazepam benzophenone	265	411	146
HO-flurazepam benzophenone	293	462	169*

* Result not consistent with proposed structure.

Table 3. Structural Confirmation of TMS Substituted Benzodiazepines. Substitution by one or two TMS groups results in the addition of 72 or 144 amu to the parent compound.

COMPOUND	MOL. WT.	MOL. ION. TMS DERIV.	OBSERVED MASS DIFFERENCE
Oxazepam-D5	291	435	144
Oxazepam	286	430	144
Nordiazepam-D5	275	347	72
Nordiazepam	270	342	72
3-OH diazepam	300	372	72
Lorazepam	320	464	144
HO-ethyl-flurazepam	332	403	72
Alpha-HA	323	395	72
Alpha-HT	358	430	72

Table 4. Structural Confirmation of TBDMS derivatives. Substitution by one or two TBDMS groups results in an increase of 114 or 228 amu to the parent compound. Base peak for all derivatives equals the mass of the molecular ion less 57.

COMPOUND	MOL. WT.	MOL. ION. TBDMS DERIV.	MASS DIFFERENCE	BASE PEAK TBDMS DERIV.
Oxazepam-D5	291	519	228	462
Oxazepam	286	514	228	457
Nordiazepam-D5	275	389	114	332
Nordiazepam	270	384	114	327
3-OH diazepam	300	414	114	357
Lorazepam	320	548	228	491
HO-ethyl-flurazepam	332	446	114	389
Alpha-HA	323	437	114	380
Alpha-HT	358	472	114	415

Table 5. Linearity and Sensitivity of Benzodiazepine-TBDMS Method.

COMPOUND	LINEARITY (ng/ml)	LIMIT OF DETECTION (ng/ml)	LIMIT OF QUANTITATION (ng/ml)
Nordiazepam-TBDMS	50--1500	1.42	3.59
Oxazepam-TBDMS	50-2000	0.80	2.27
Temazepam-TBDMS	50-2000	0.24	0.66
Lorazepam-TBDMS	50-2000	0.18	0.46
HO-ethyl-flurazepam-TBDMS	50-1000	0.03	0.12
Alpha-HA-TBDMS	50-2000	1.20	3.65

Table 6. Precison of Benzodiazepine-TBDMS Method.

COMPOUND	WITHIN-RUN C.V.% 100 ng/ml (n=15)	BETWEEN-RUN C.V.% 100 ng/ml (n=6)	BETWEEN-RUN C.V.% 1000 ng/ml (n=6)
Nordiazepam	4.82	2.32	0.98
Oxazepam	3.54	5.22	3.98
Temazepam	6.18	6.63	4.98
Lorazepam	5.25	8.28	3.22
HO-ethyl-flurazepam	5.73	8.08	10.52
Alpha-HA	2.21	8.24	6.49

Table 7. Selected Ions for Benzodiazepine-TBDMS Method.

COMPOUND	QUANTITATIVE ION	PEAK QUALIFIER(S)
Nordiazepam-D5-TBDMS	332	334
Nordiazepam-TBDMS	327	328, 329
Oxazepam-D5-TBDMS	462	464
Oxazepam-TBDMS	457	458, 459
Temazepam-TBDMS	357	359, 283
Lorazepam-TBDMS	491	493, 513
HO-ethyl-flurazepam-TBDMS	389	390, 391
Alpha-HA-TBDMS	381	382, 383

Table 8. GC/MS results of 66 RIA positive human urine specimens.

	NUMBER OF SPECIMENS BY DRUG GROUP AND CONCENTRATION RANGE (NG/ML)					
	50-100	100-200	200-500	500-1000	>1000	TOTAL
Nordiazepam	15	8	8	3	0	34
Oxazepam	6	12	12	9	9	48
Temazepam	10	10	8	3	2	33
Lorazepam	0	0	1	0	0	1
HO-ethyl-flurazepam	0	1	3	4	3	11
Alpha-HA	1	0	0	1	1	3

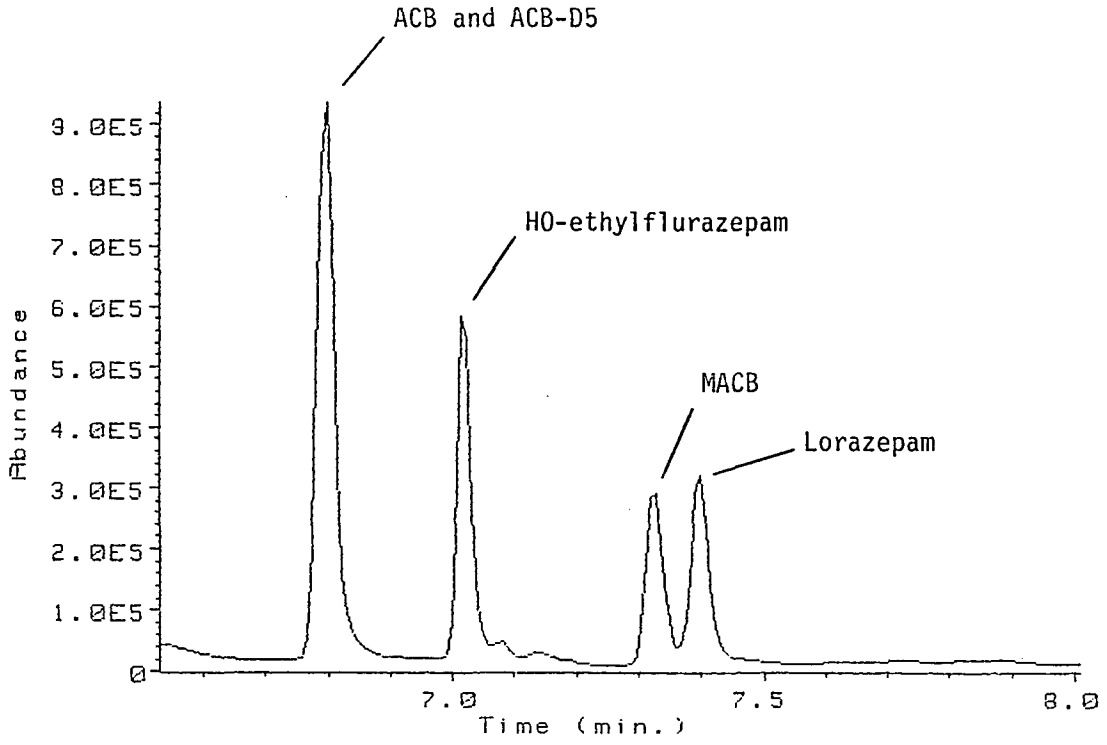

Figure 5. Selected ion chromatograph of mixed benzophenone-PFP derivatives. (200 ng/ml extracted urine calibration standard) Stationary phase; J&W DB-17, 50% phenyl methylsilicone

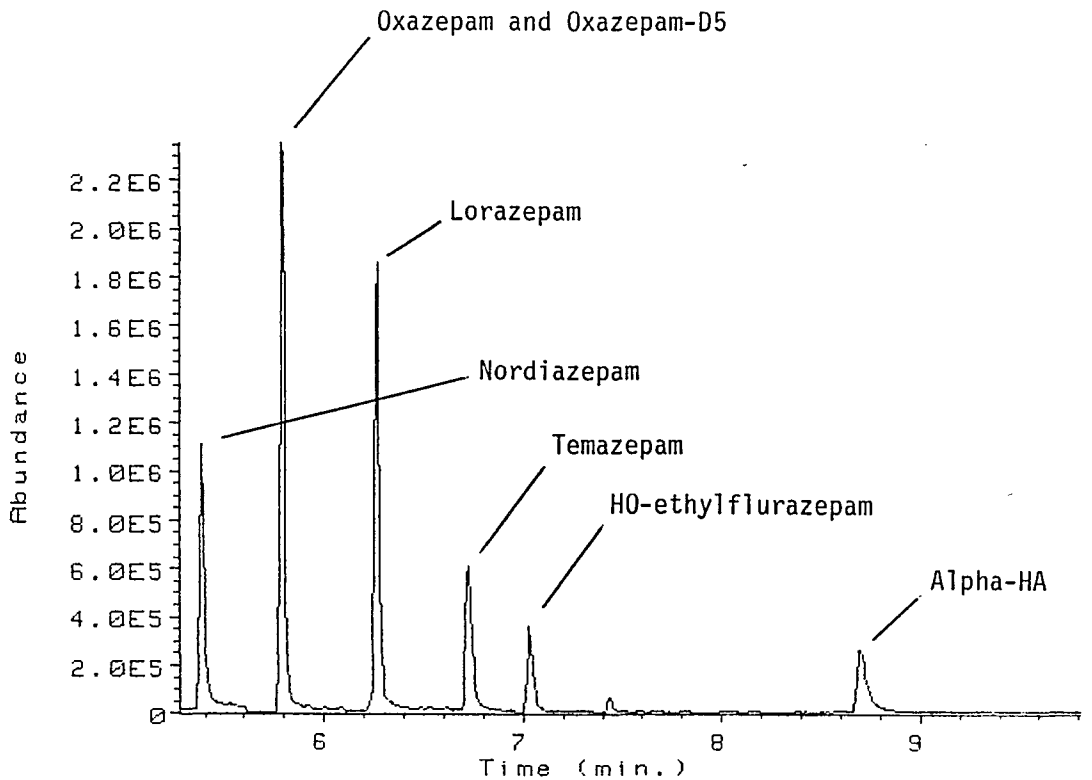

Figure 6. Selected ion chromatograph of mixed benzodiazepine-TMS derivatives. (200 ng/ml extracted urine calibration standard) Stationary phase; HP Ultra 1, methylsilicone

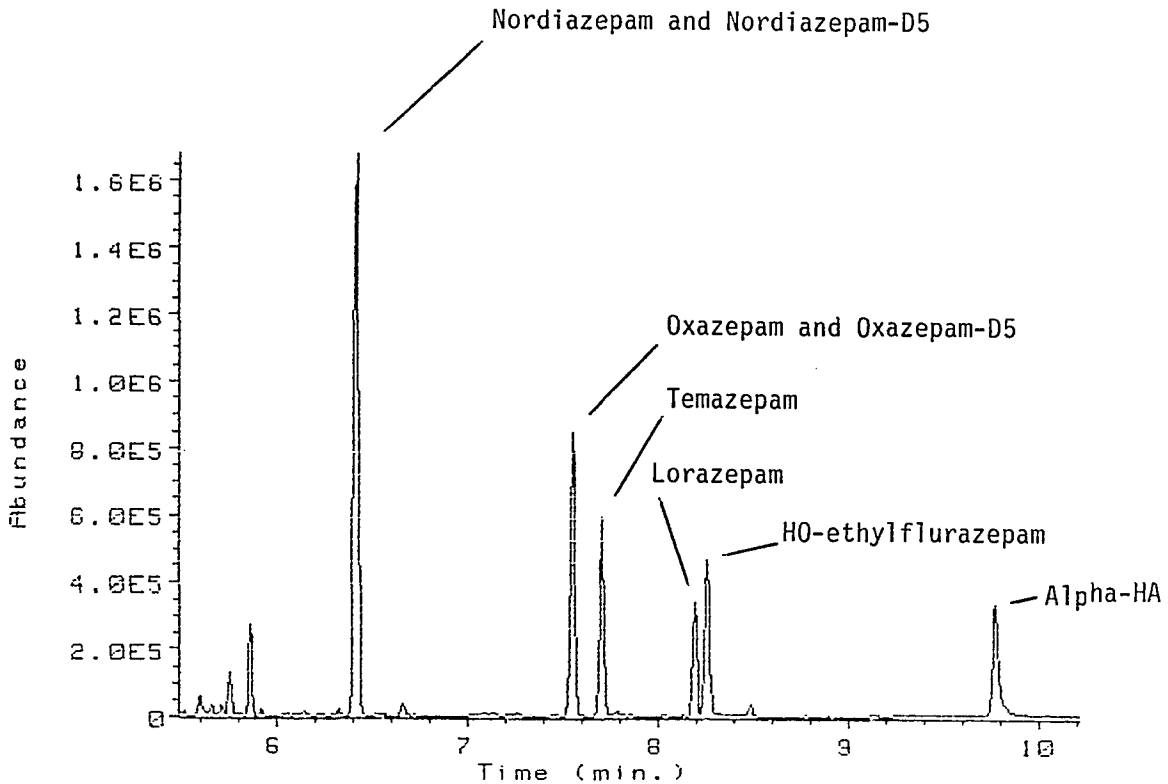

Figure 7. Selected ion chromatograph of mixed benzodiazepine-TBDMS derivatives. Extracted 200 ng/ml urine calibration standard. Stationary phase; HP Ultra 1 (methylsilicone).

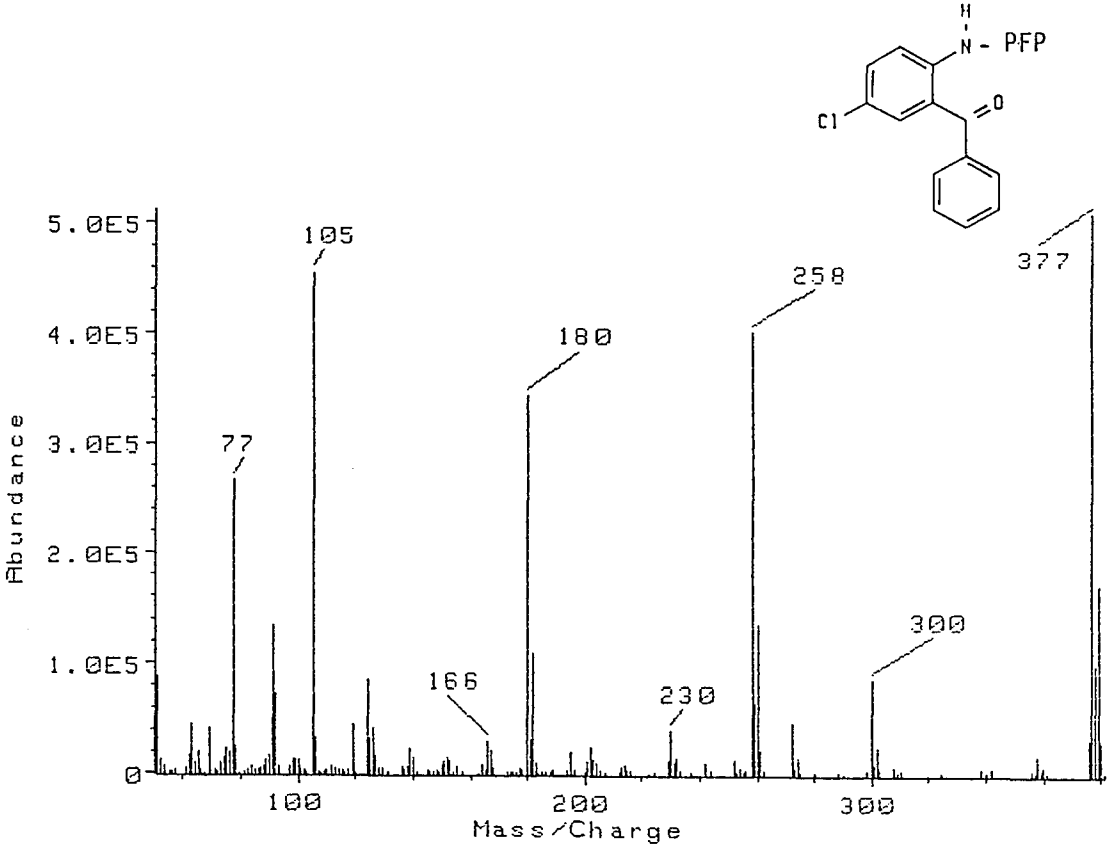

Figure 8a. Mass spectrum of aminochlorobenzophenone-PFP

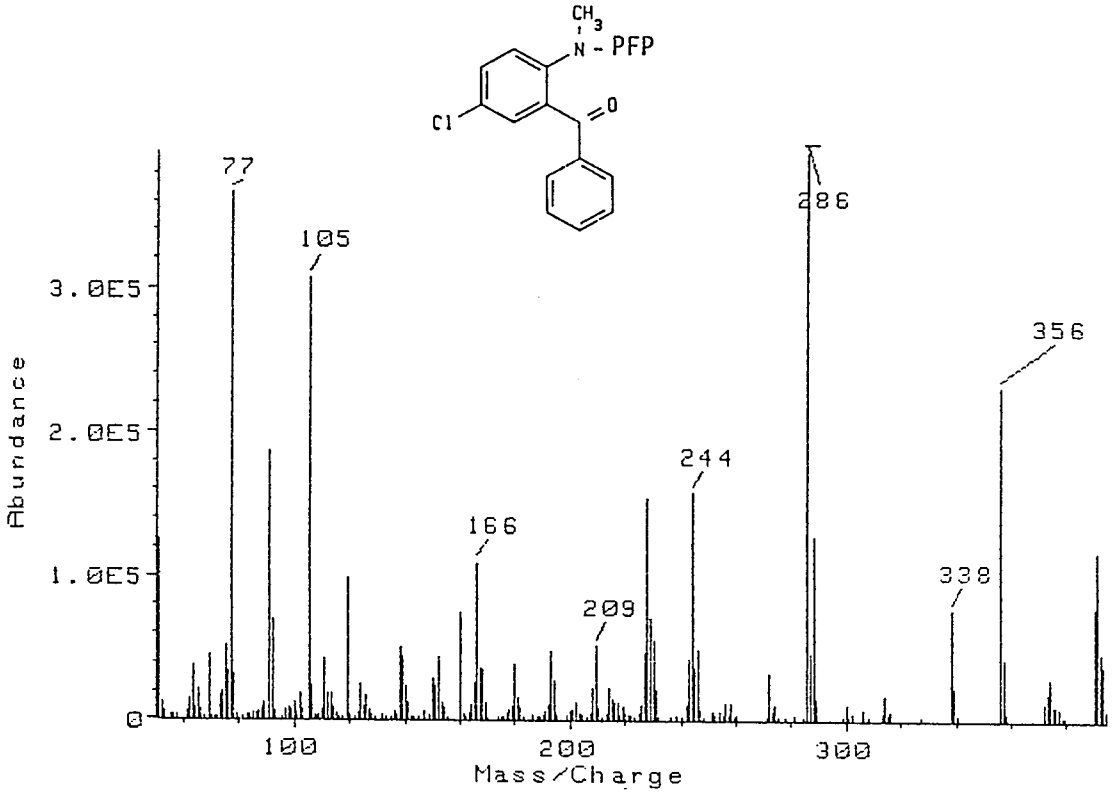

Figure 8b. Mass spectrum of methylaminochlorobenzophenone-PFP

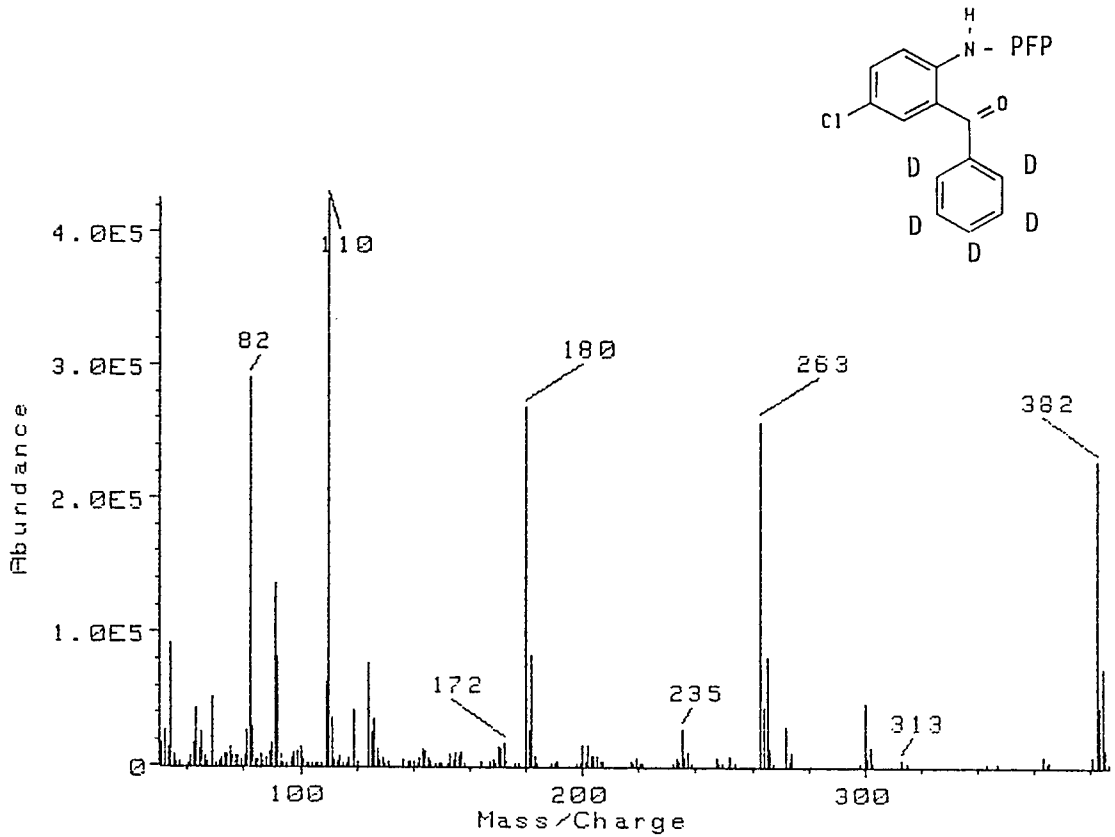

Figure 8c. Mass spectrum of aminochlorobenzophenone-D5-PFP

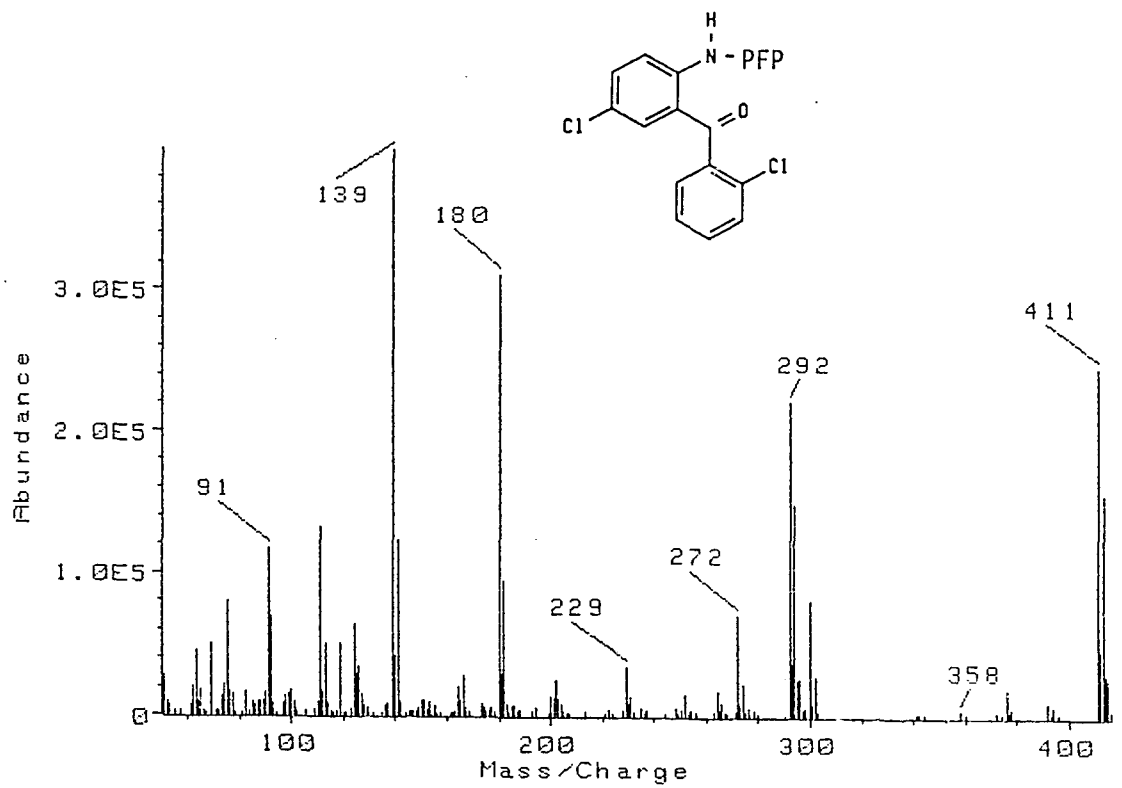

Figure 8d. Mass spectrum of lorazepam-benzophenone-PFP

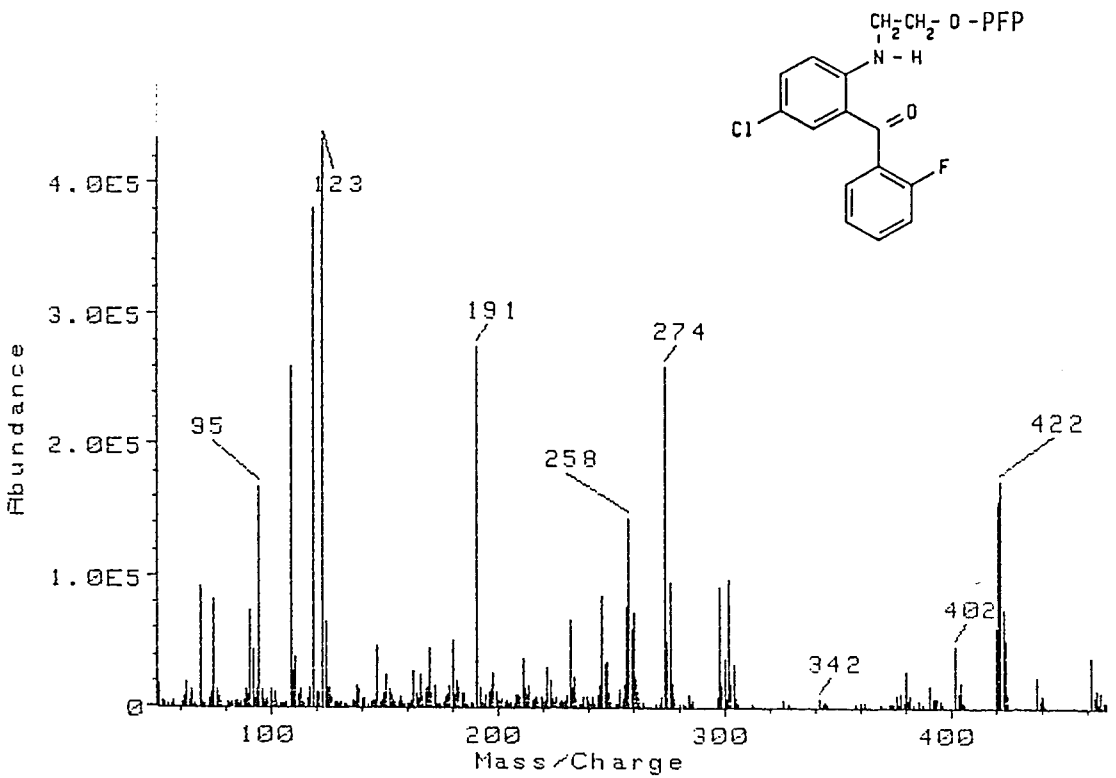

Figure 8e. Mass spectrum of HO-ethylflurazepam benzophenone-PFP

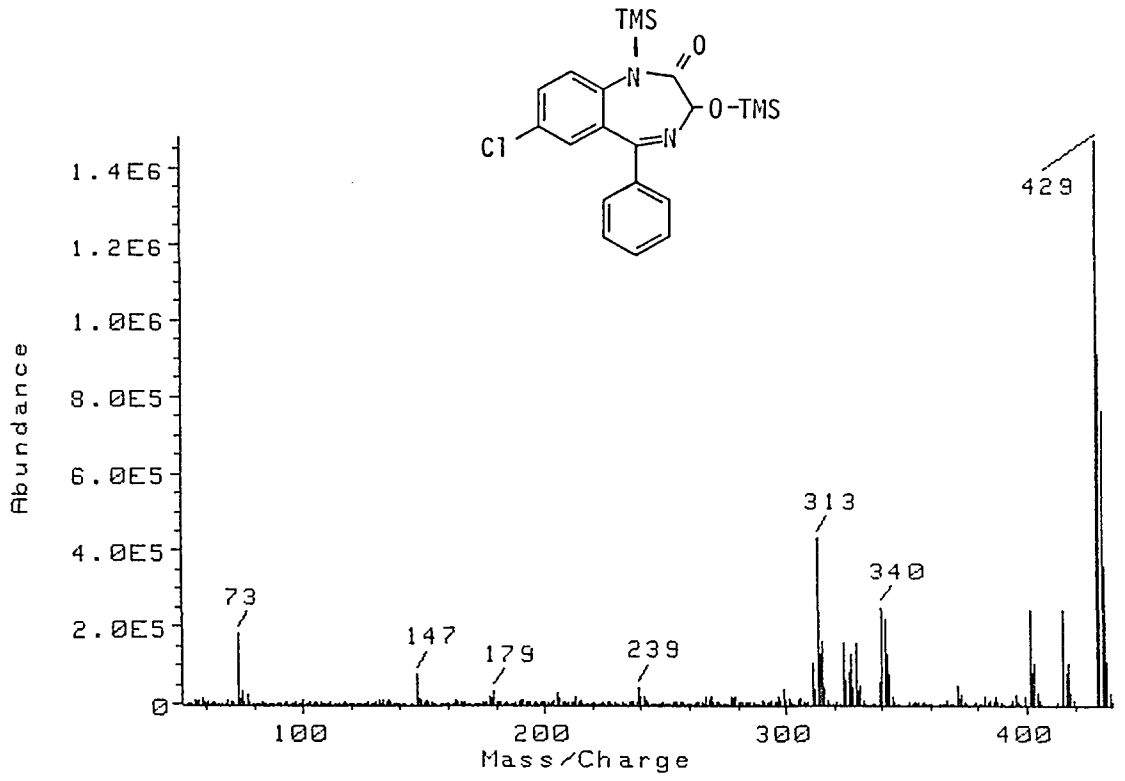

Figure 9a. Mass spectrum of oxazepam-TMS

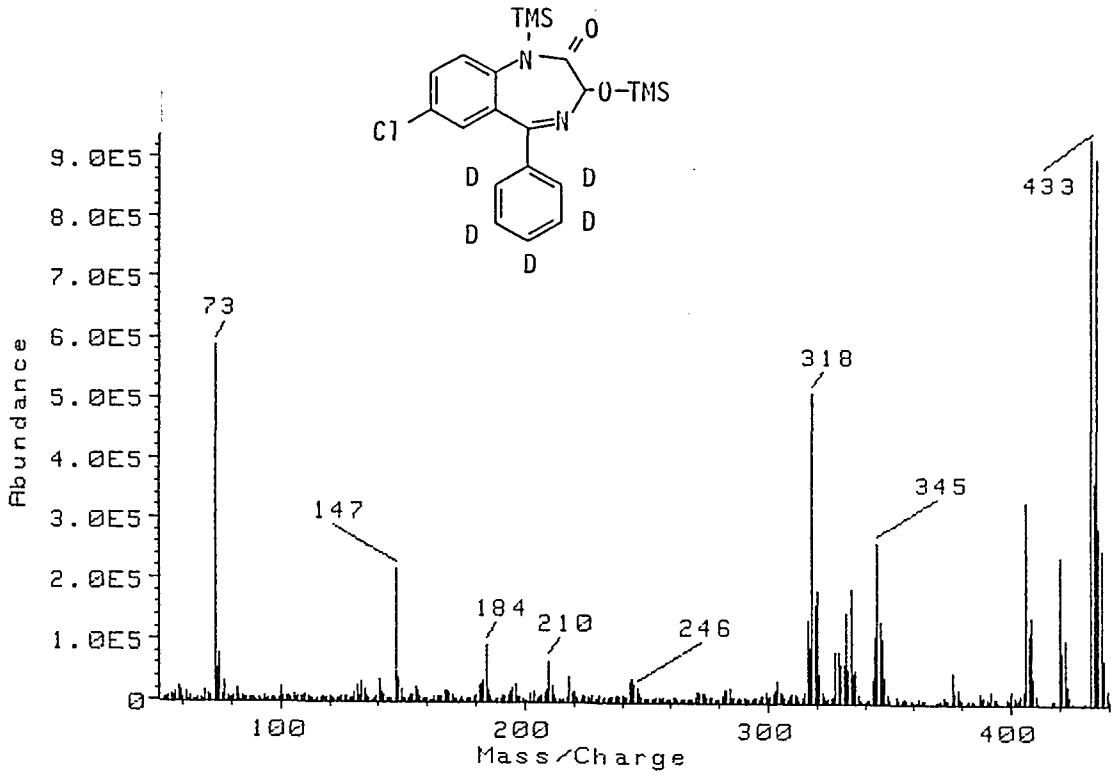

Figure 9b. Mass spectrum of oxazepam-D5-TMS

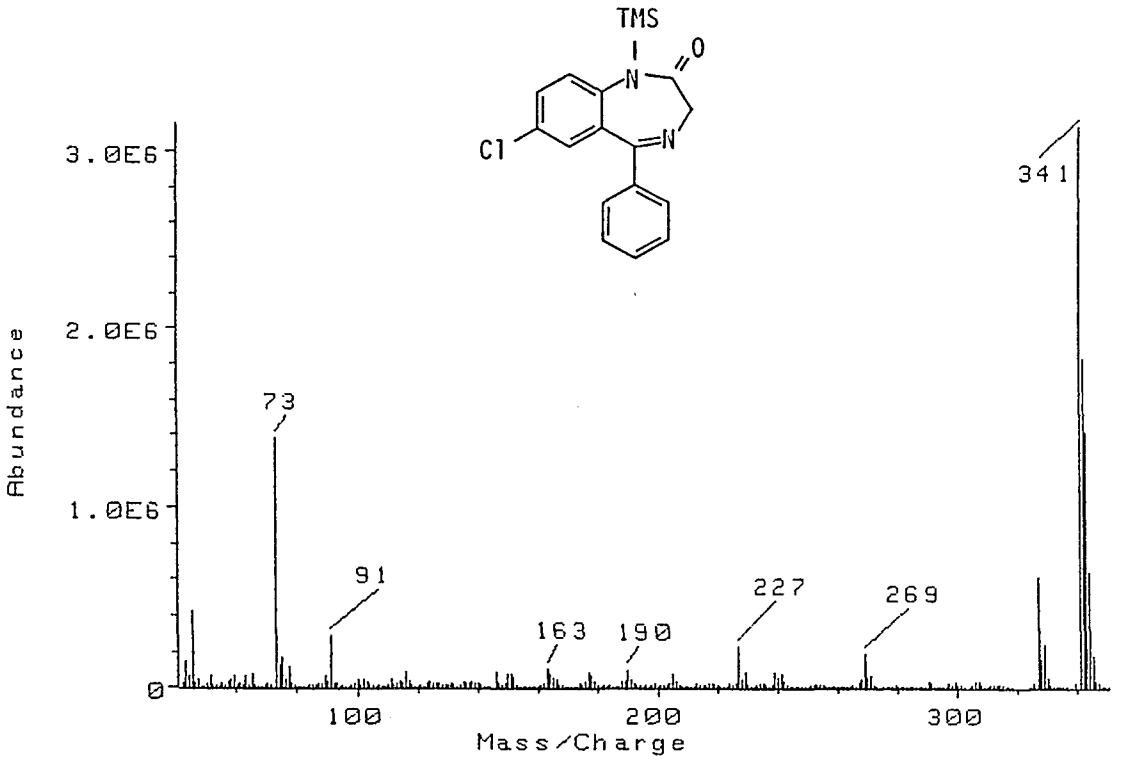

Figure 9c. Mass spectrum of nordiazepam-TMS

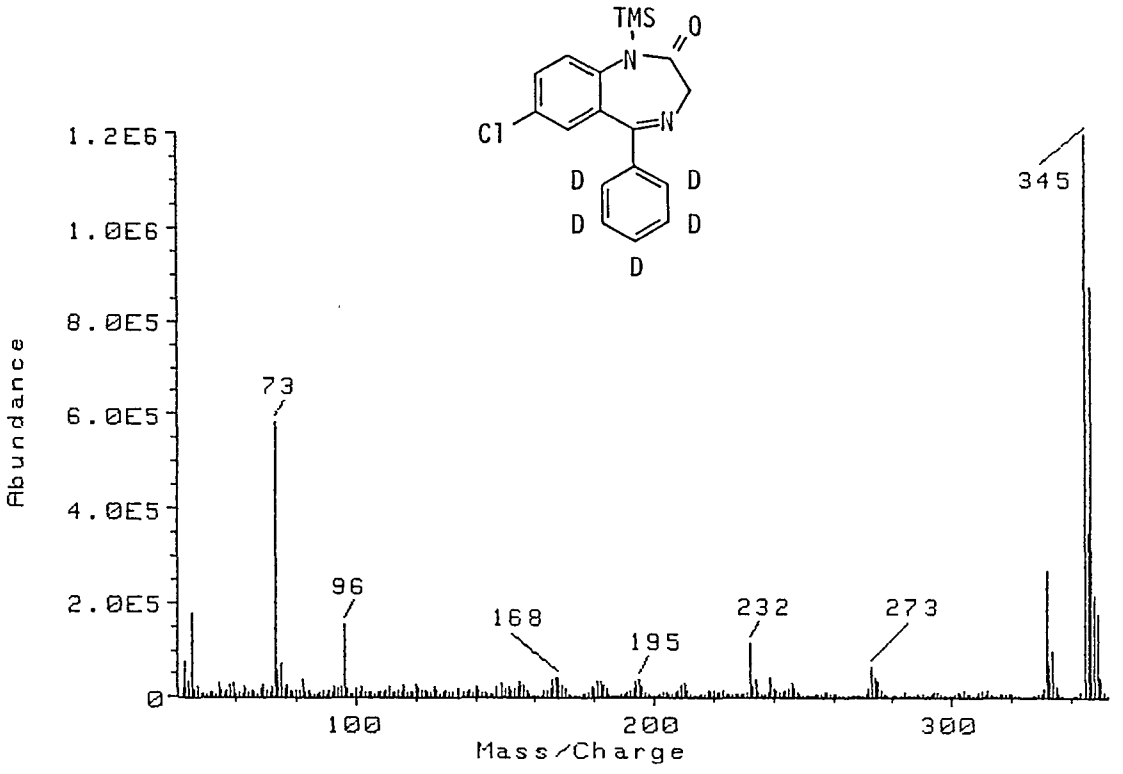

Figure 9d. Mass spectrum of nordiazepam-D5-TMS

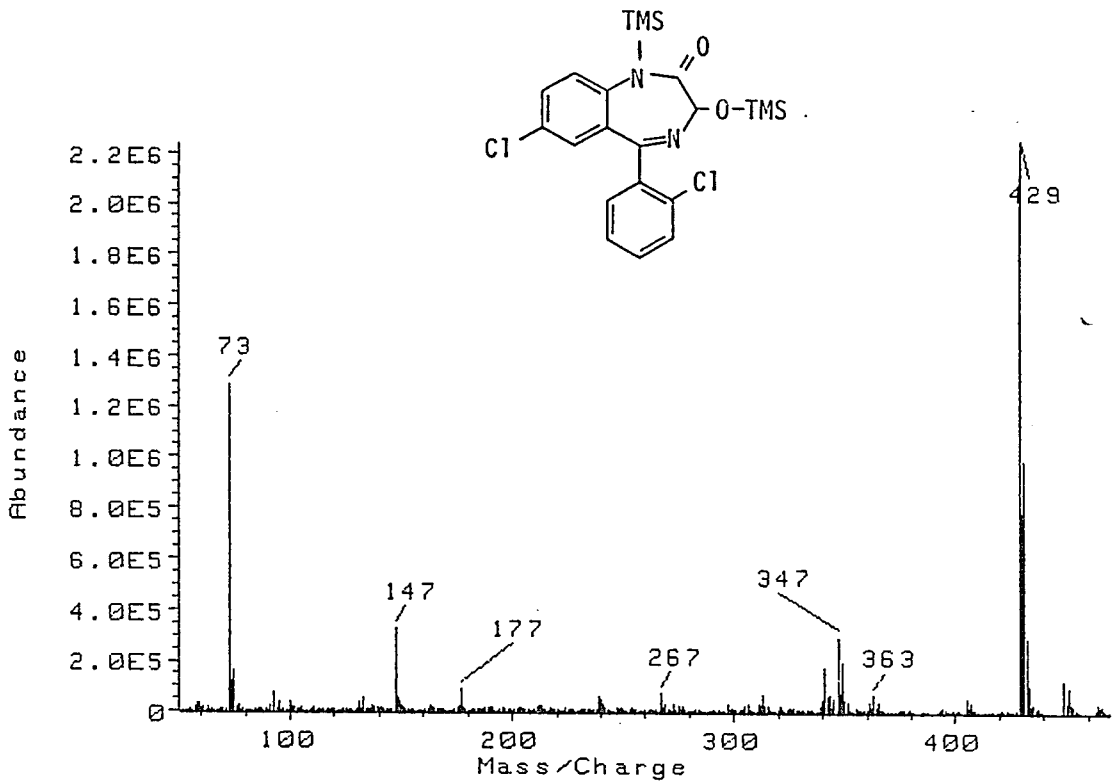

Figure 9e. Mass spectrum of lorazepam-TMS

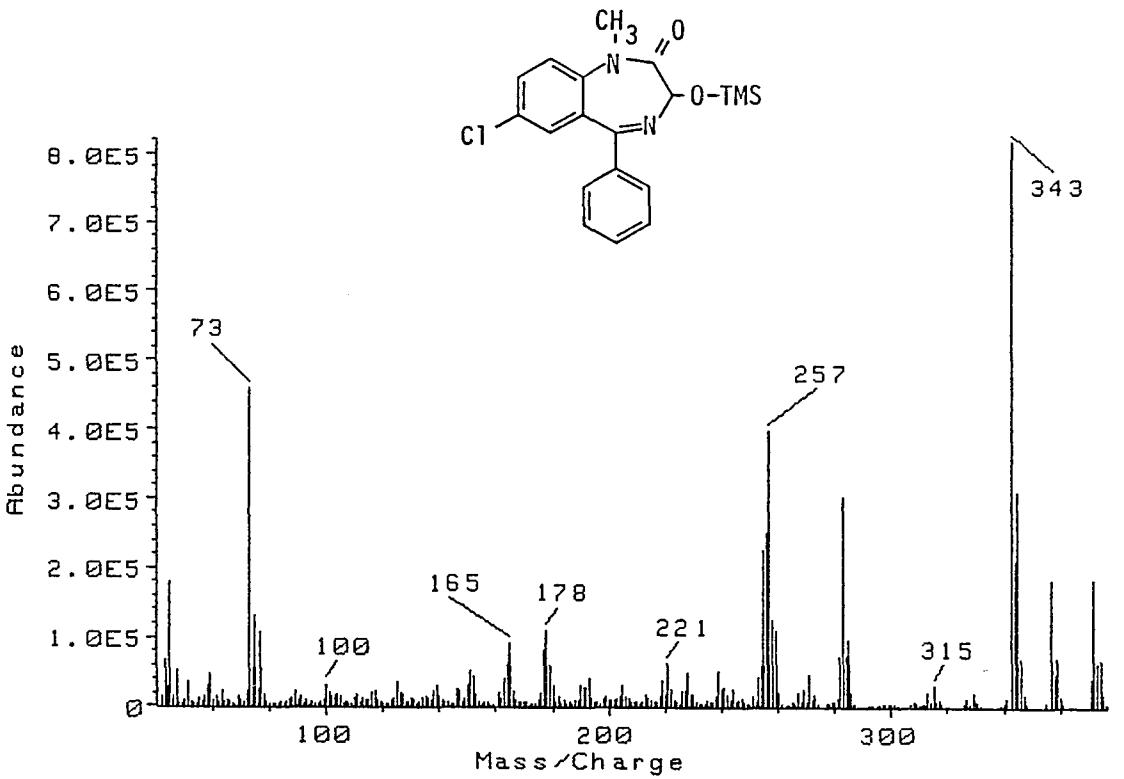

Figure 9f. Mass spectrum of 3-OH diazepam-TMS

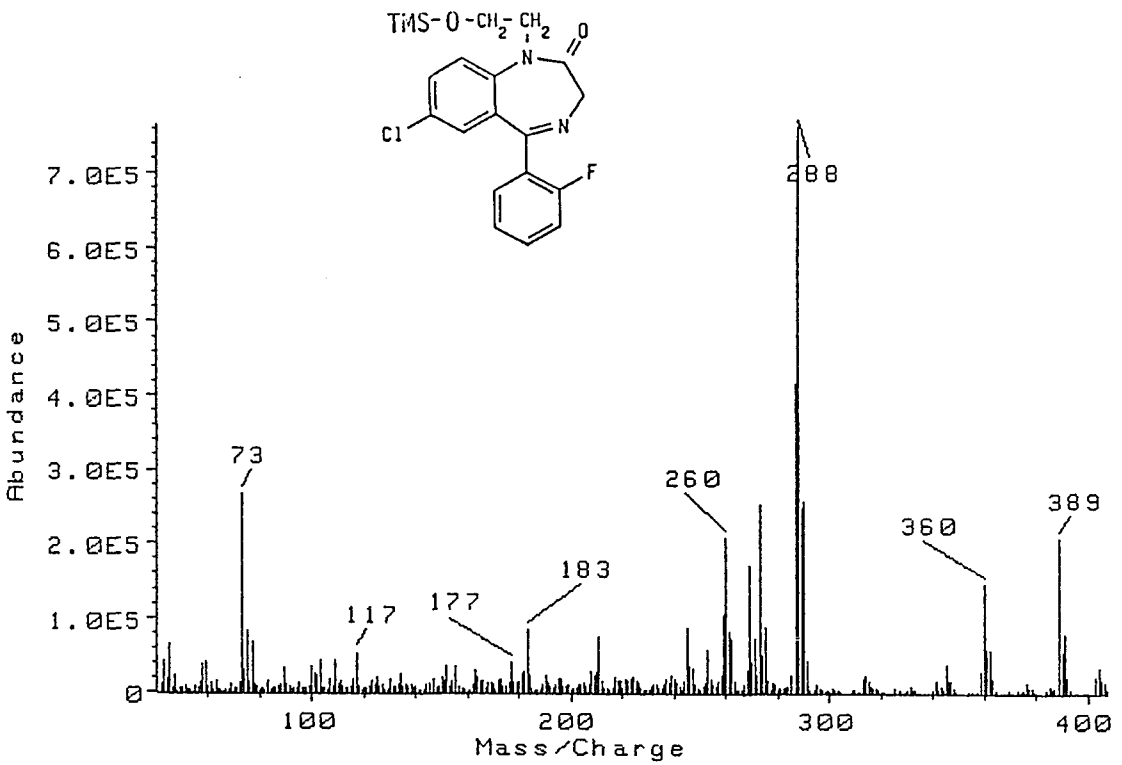

Figure 9g. Mass spectrum of HO-ethylflurazepam-TMS

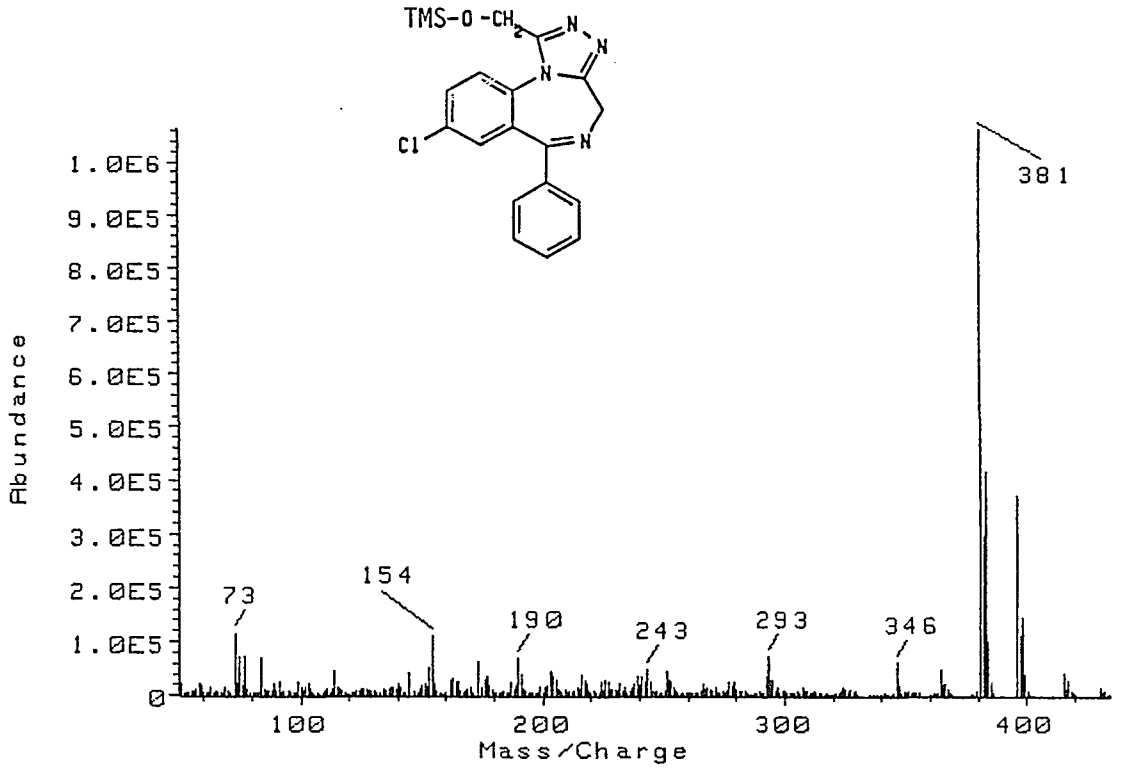

Figure 9h. Mass spectrum of alpha hydroxyalprazolam-TMS

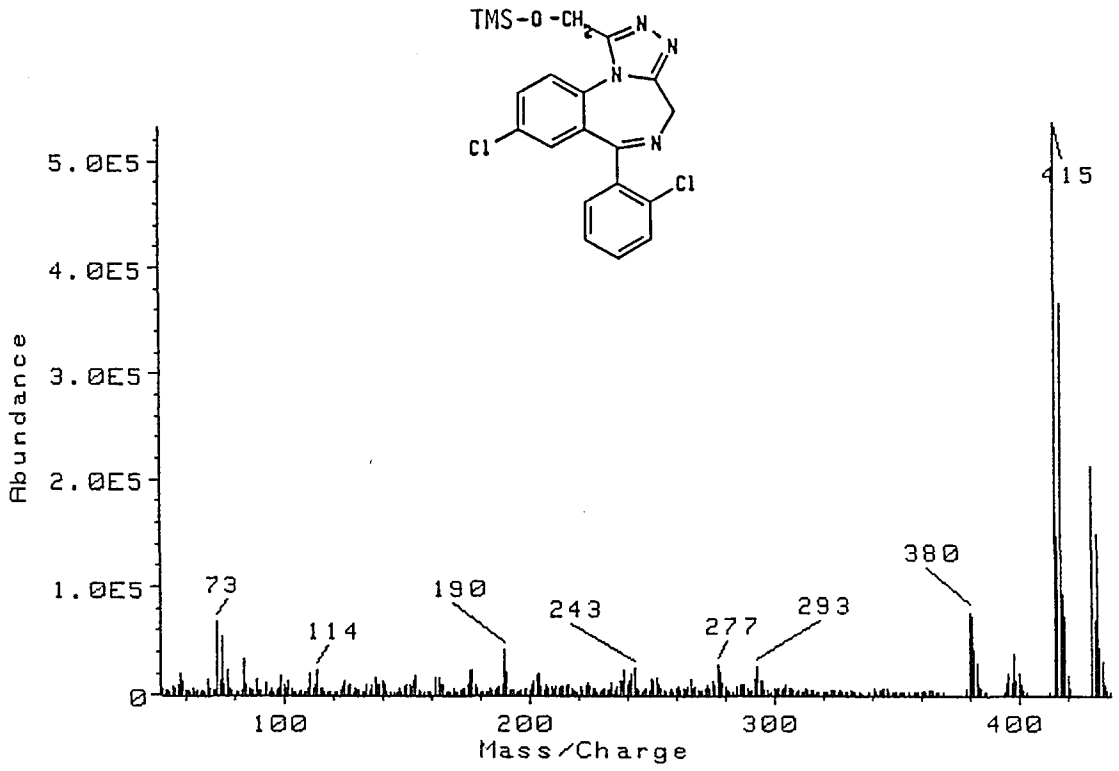

Figure 9i. Mass spectrum of alpha hydroxytriazolam-TMS

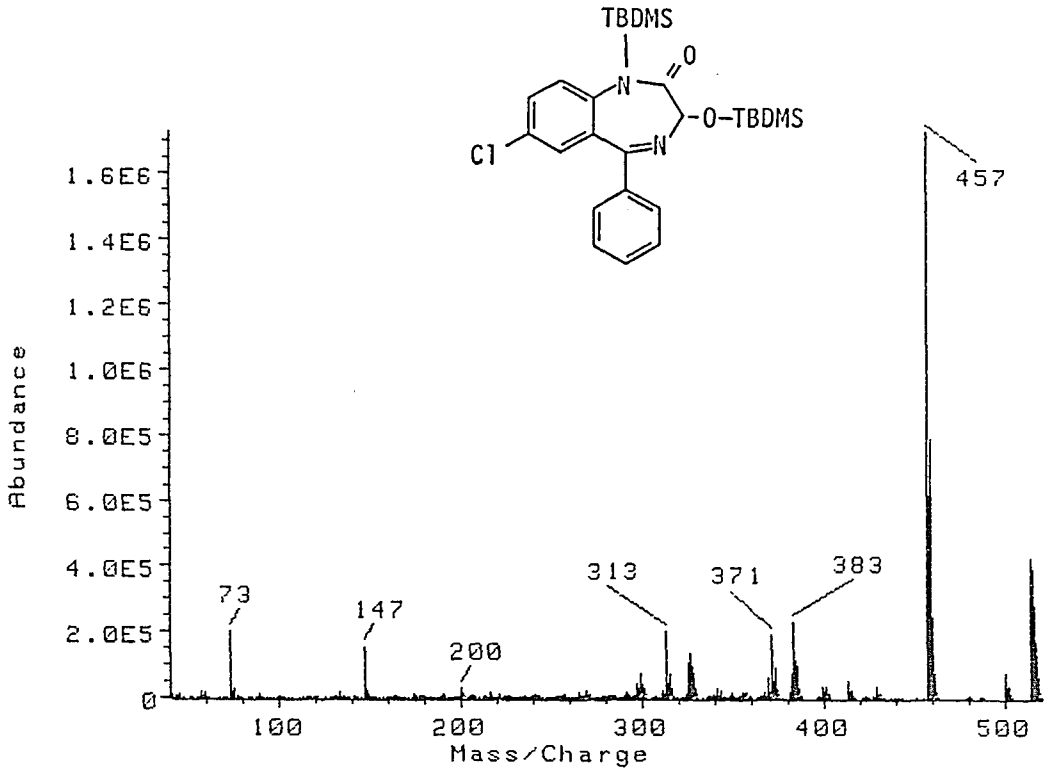

Figure 10a. Mass spectrum of oxazepam-TBDMS

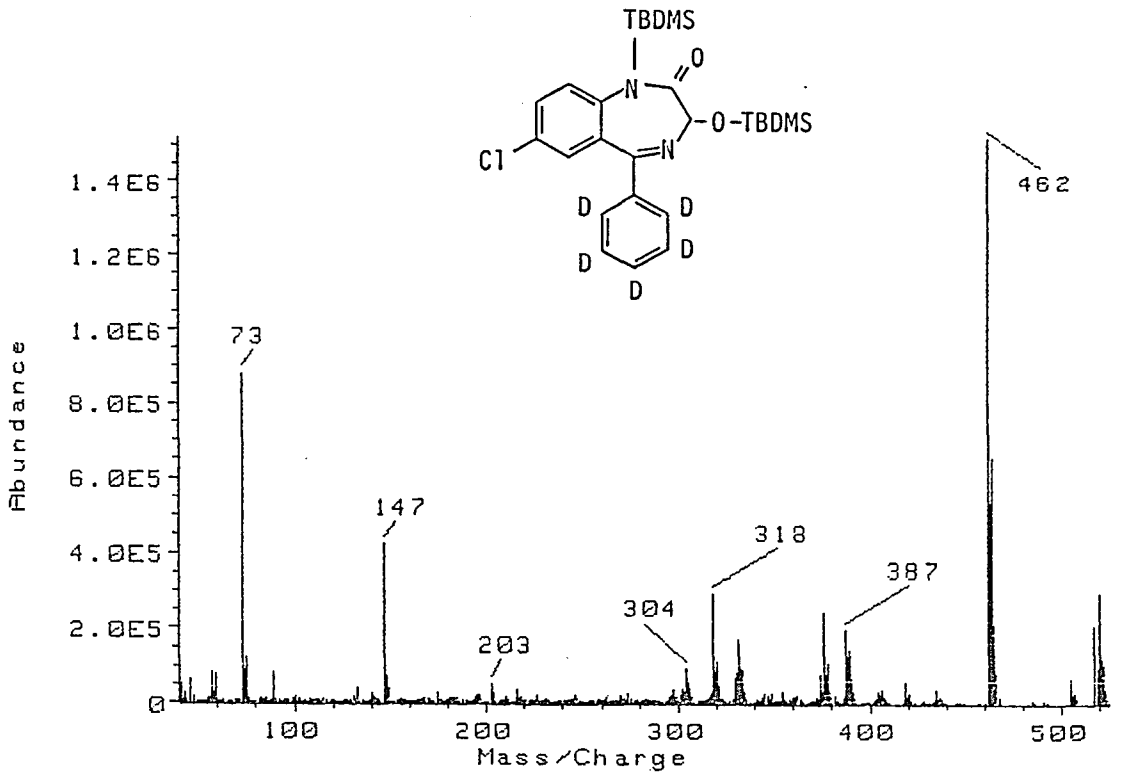

Figure 10b. Mass spectrum of oxazepam-D5-TBDMS

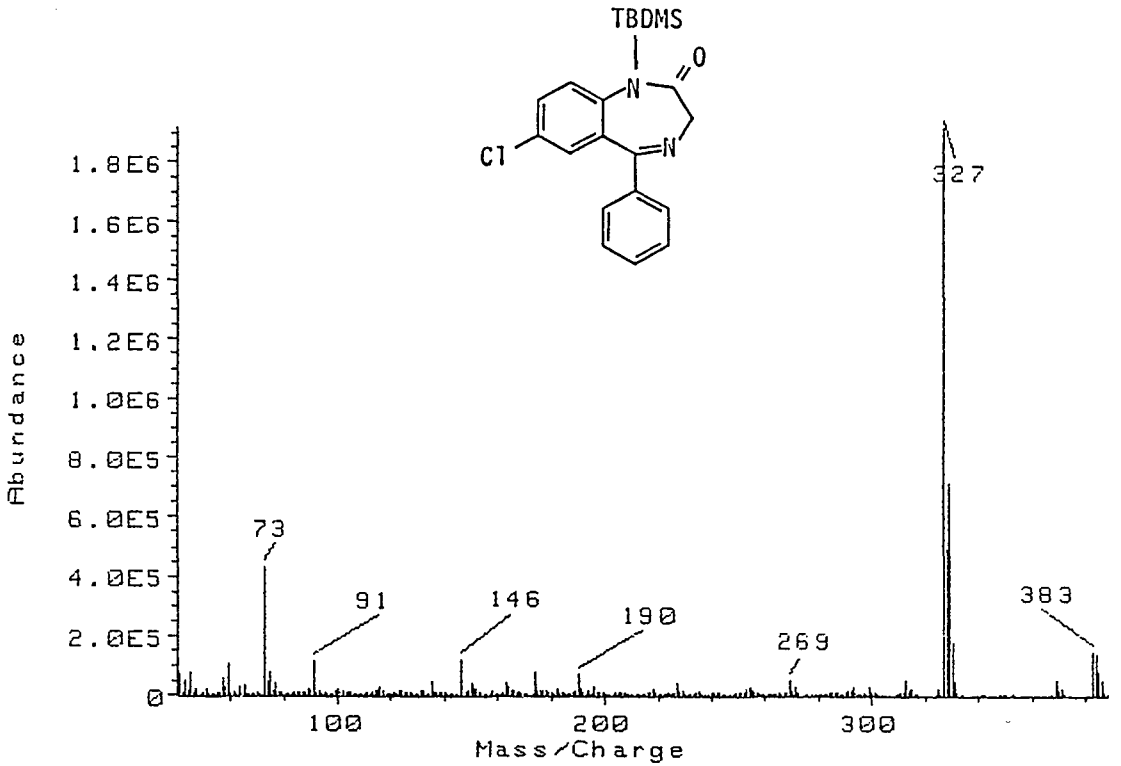

Figure 10c. Mass spectrum of nordiazepam-TBDMS

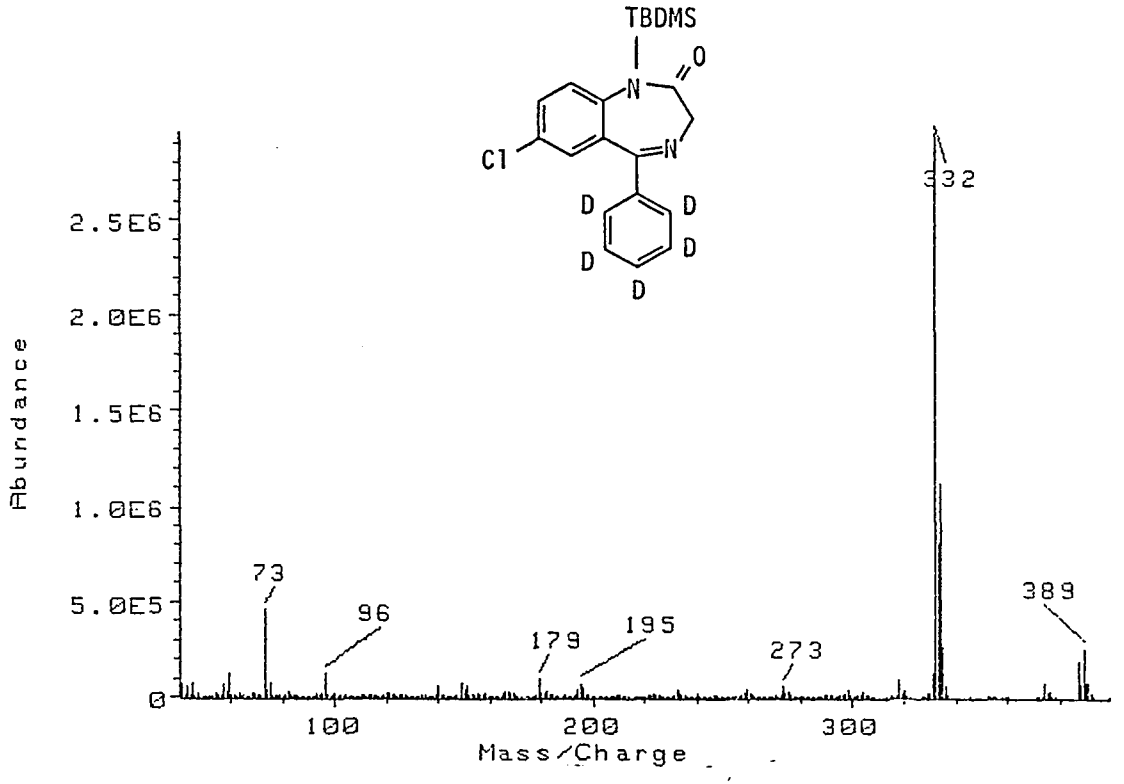

Figure 10d. Mass spectrum of nordiazepam-D5-TBDMS

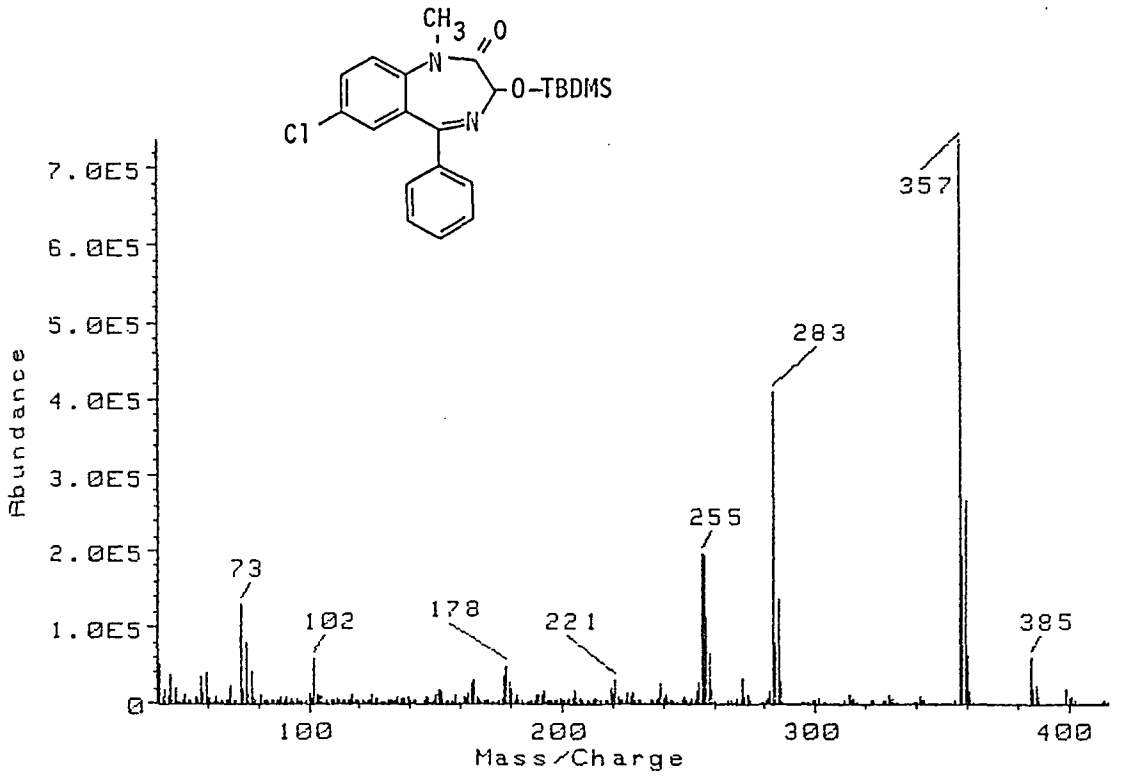

Figure 10e. Mass spectrum of 3-OH diazepam-TBDMS

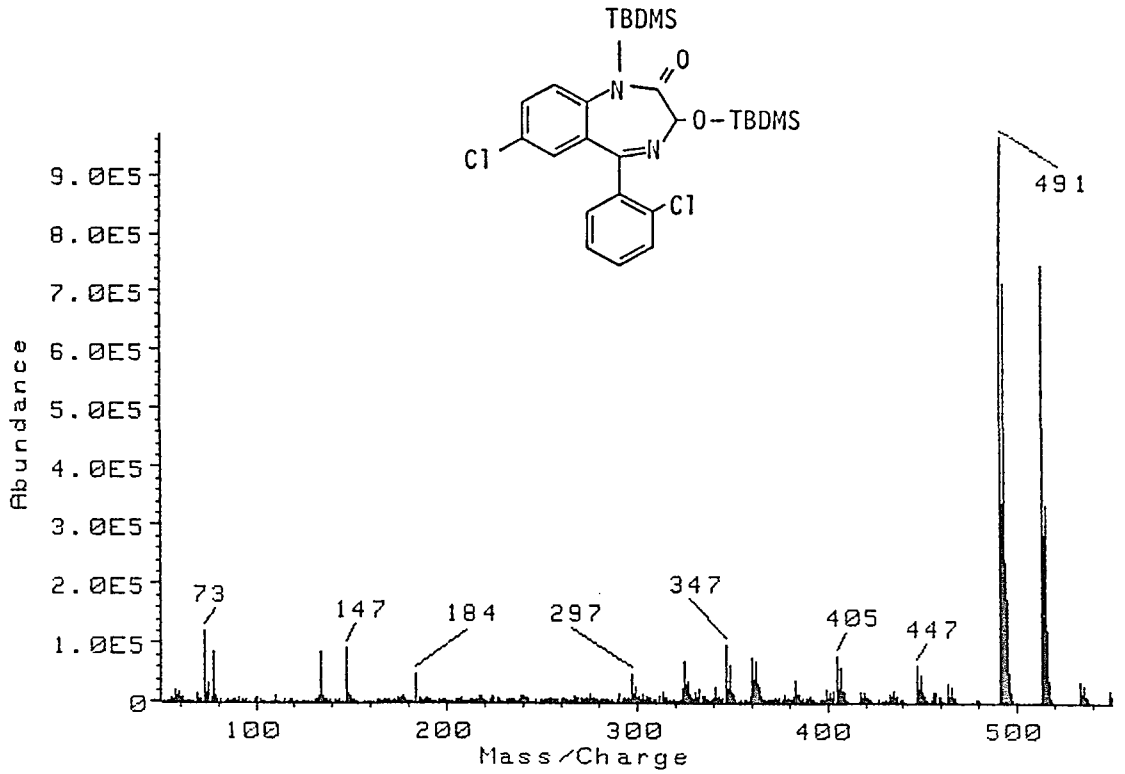

Figure 10f. Mass spectrum of lorazepam-TBDMS

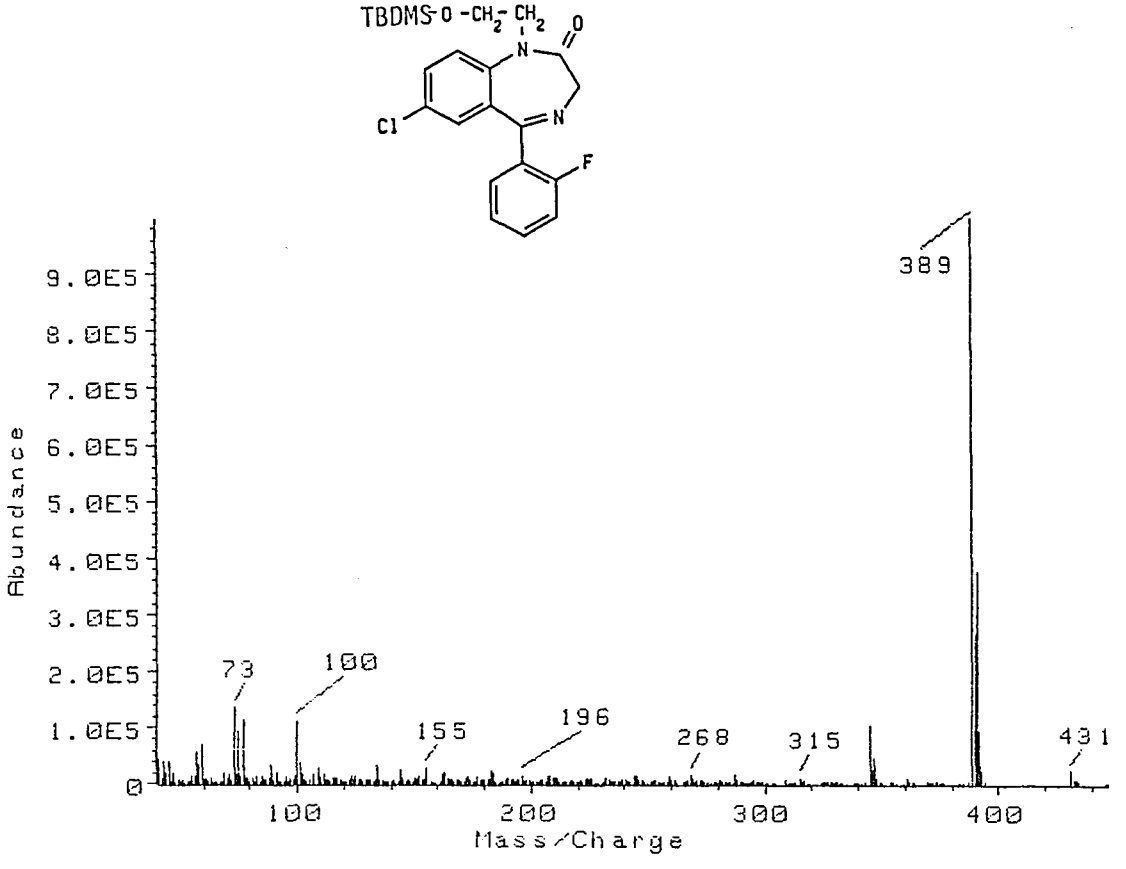

Figure 10g. Mass spectrum of HO-ethylflurazepam-TBDMS

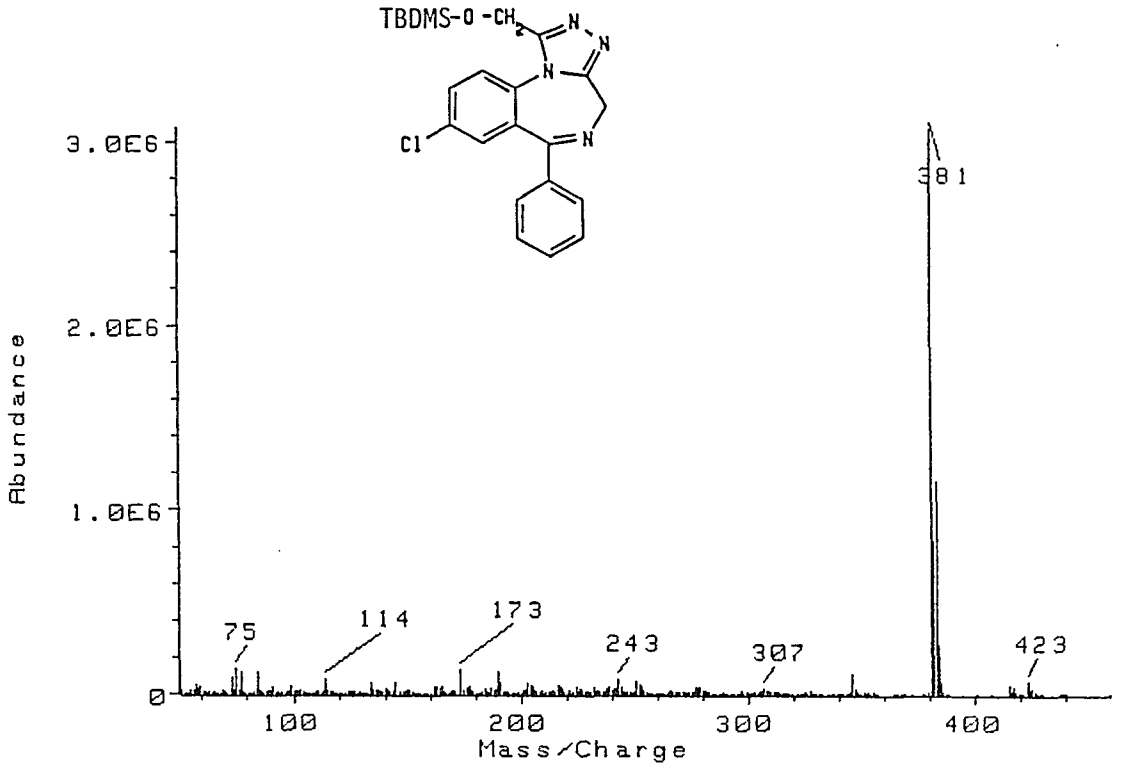

Figure 10h. Mass spectrum of alpha hydroxyalprazolam-TBDMS

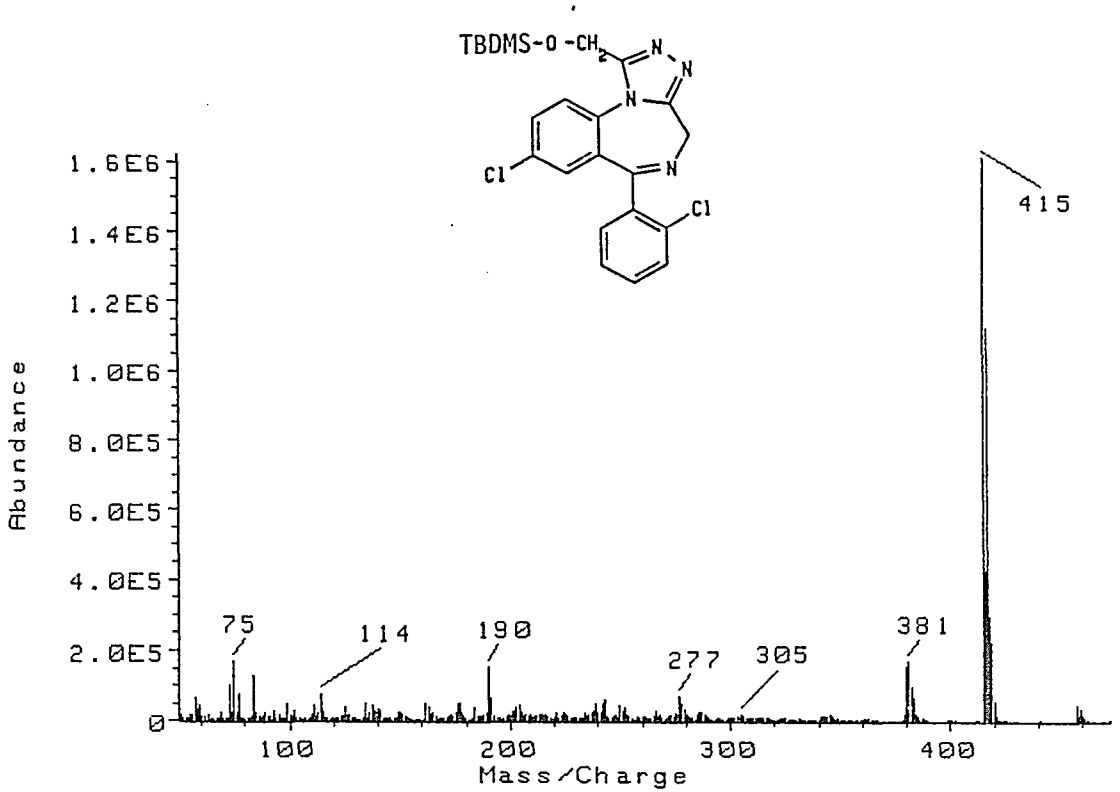

Figure 10i. Mass spectrum of alpha hydroxytriazolam-TBDMS

DISCUSSION

Of the three benzodiazepine derivatives tested, the TBDMS derivatives were found best suited for use in a comprehensive GC/MS assay, although certain advantages were found for each. The benzophenone-PFP compounds were easy to prepare, chromatographed well, and allowed for a convenient and straight-forward analysis. Unfortunately, the usefulness of this approach is limited by the fact that the alprazolam and triazolam metabolites do not form PFP derivatives and by difficulties in the interpretation of results. Much of the ambiguity relating the the interpretation of benzophenone results is eliminated by the use of either TMS or TBDMS derivatives. All metabolites tested, including alpha-HA and alpha-HT reacted to form these derivatives. This allowed for the analysis of most benzodiazepine metabolites in a comprehensive panel. The TMS derivatives, however, were found to be unstable and the preliminary assay based on this derivative did not yield the desired precision. TBDMS derivatives were stable and gave adequate precision but were less volatile than the corresponding TMS derivatives.

Hydrolysis of urinary benzodiazepines to benzophenones, followed by acylation is an established approach for the simultaneous analysis of these compounds (Sanchez, et al. 1976, Pfleger, et al. 1985, Maurer, et al. 1987). This analytical approach was the basis of the benzophenone-PFP method. Unfortunately, this method could not be extended to include the analysis of alpha-HA and alpha-HT. The inability of alpha-HA to react with pentafluoropropionic anhydride was confirmed by

Joern, et al. (1987) who found that both pentafluoropropionic anhydride (PFPA) and trifluoroacetic anhydride (TFA) were unsuitable for the derivatization of this metabolite.

Although nonreactive with PFPA and TFA, references were found indicating that alpha-HA and alpha-HT could be chromatographed as acyl derivatives (Joern, et al. 1987, Pfleger, et al. 1985). It seemed reasonable that by the substitution of acetic anhydride for PFPA as a derivatization reagent, both compounds could be included in the benzophenone method. Both metabolites are resistant to acid hydrolysis (Wong 1984), so unlike the other compounds in the assay, they would not form benzophenones and would be identified as intact metabolites. This derivative was also found unsuitable since the derivatives formed chromatographed poorly in the GC/MS although good chromatography was obtained with a GC. It seems likely that the chromatographic problems encountered with these compounds in the GC/MS system but not the GC system, are related to either the negative effect of the MS vacuum on the distal end of the capillary column or the chromatographic dead space in the GC/MS interface. The incorporation of these metabolites into a benzophenone assay is theoretically possible with design improvements to the GC/MS system. Although the triazolobenzodiazepines were not amenable to this approach, it did prove to be a reasonably good technique for the remaining analytes.

The preliminary benzophenone-PFP method was adopted by Poisonlab Inc. for the confirmation of benzodiazepines in RIA positive urines. The method was found capable of consistently confirming RIA positives at 100 ng/ml using a GC/MS limit of analysis at the same concentration.

The ability of the benzophenone method to confirm RIA positives at the same concentration, points to one of the basic advantages of this analytical approach. Acid hydrolysis of a specimen containing oxazepam and nordiazepam each at a concentration of 50 ng/ml would result in the formation of a single benzophenone, ACB, at approximately 100 ng/ml. (Verebey, et al. 1982, Maurer, et al. 1980). The same specimen would be expected to register a positive by RIA since it contains a total of 100 ng/ml immunoreactive benzodiazepines. For many specimens, a better correlation between RIA and GC/MS results would be expected using the benzophenone method as opposed to other GC/MS methods that test for the intact metabolites. The results of this work confirmed this expectation since three specimens that had tested positive using the benzophenone method with a 100 ng/ml limit of analysis, tested below this level using the TBDMS method.

Other advantages were found in the reliability and simplicity of the procedure. The initial acid hydrolysis step serves both to free urinary conjugates and to form benzophenones. The use of hexane as an extraction solvent results in interference free extracts. Finally, the derivatives that result from the reaction with PFPA were found to be quite stable, have chromatographic properties superior to the underivatized benzophenones, and produce useful high molecular weight ions in the MS.

As the laboratory gained more experience with the procedure, problems arose in the reporting and interpretation of results. When ACB and MACB were identified, they were reported as "benzodiazepines (ACB and MACB) identified." This often required a rather long explanation of

what these benzodiazepines represent in terms of the drug ingested. Another option considered was to simply report "benzodiazepines identified". This idea was discarded because it was felt that more specificity is required in the reporting of forensic results. This became even more clear when the benzophenone method was used in the analysis of police department cases. The prosecuting attorneys involved in these cases sometimes objected to the reporting format because it was felt that a specific drug or metabolite must be indicated on the toxicology report in order to issue a charge.

Benzodiazepine positive results presented as benzophenone derivatives has been and will continue to be admissible evidence in civil and criminal court. However, the expert witness often finds it difficult to provide a simple explanation or interpretation of the result to a judge or jury. For this reason, the forensic toxicologist is to a certain extent placed at a disadvantage by the use of this reporting format.

TMS derivatives have long been used in the analysis of these compounds (de Silva, et al. 1976, Coassello, et al. 1983). In the thesis work they were found to be somewhat erratic and less than satisfactory in terms of precision for the triazolobenzodiazepine metabolites. It is interesting to note that Mule, et al. (1989), reported the same difficulties with these troublesome compounds. This author reported C.V. data of 19% at 2000 ng/ml for the alpha-HA metabolite using a TMS derivative. This rather poor precision was seen also in this work for the same derivative. Our C.V. for alpha-HA at 100 ng/ml using the same derivative was found to be 20%. Precision reported

by Mule for the other metabolites was good. Use of these derivatives might be an attractive alternative if the stability problem could be overcome.

In most respects, the data generated using the benzodiazepine-TBDMS method indicate that the procedure will be useful in its intended application, that is, the confirmation of RIA positive urine specimens. The analysis of 66 specimens divided into six daily runs, found the method reliable in day to day use, relatively easy to use and essentially interference free. More important, nearly 100% of the RIA positive urines were confirmed using a 100 ng/ml GC/MS cutoff. Two shortcomings of the procedure are it's marginal precision for alpha-HA and it's inability to test for alpha-HT.

Another approach might overcome these problems. Joern, et al. (1987) was able to confirm alprazolam use by the detection of its benzophenone metabolite. Although it is very difficult to hydrolyze this metabolite to the benzophenone in vitro, biotransformation of this compound to the benzophenone by the liver is significant (Moffat 1986). The retention time for this metabolite is shorter than alpha-HA and would likely be more suitable to GC/MS analysis. Triazolam is also eliminated to a significant extent as a benzophenone and might also be confirmed in this way. Unfortunately, neither compound could be obtained for this work. These benzophenone metabolites represent a smaller percentage of the total metabolites eliminated, however, and a lower confirmation limit of perhaps 50 ng/ml would be required to confirm use of these drugs. Both benzophenone metabolites posses reactive hydroxyl groups that would be expected to react with MSTBDMS.

Use of MSTBDMS in place of various TMS donating reagents is a new approach to the analysis of these compounds. Unlike TMS reagents, MSTBDMS is known to react with many compounds at room temperature and form derivatives that are stable for a period of days. Disadvantages of this compound are it's inability to react easily with primary amines and the decreased volatility of the reaction products (Mawhinney, et al, 1982). MSTBDMS, therefore does not represent an alternative for all TMS applications but would likely be found useful in the analysis of hydroxylated and carboxylated compounds. Because TBDMS derivatives fragment in a predicable fashion in the MS, MSTBDMS facilitates molecular weight determinations.

Regardless of the derivative used, the interpretation of results is problematic and warrants a conservative approach. Toxicologists are frequently asked to estimate the lenght of time a benzodiazepine might be detectable in urine and time of last use. The factors effecting detectability are many and include the dose, sensitivity of the assay, the half life of the drug or its metabolite, whether the use was chronic or acute and the degree of body fat of the subject tested. It may be possible to detect the metabolites of diazepam, chlordiazepoxide and flurazepam for weeks or perhaps even months after use (Hawks 1986). These metabolites are lipophilic, have long half lives and their kinetics approximate three compartment models (Greenblatt, et al. 1978, 1984, Guentert 1984). A similar situation exists in the testing in urine for the major metabolite of marijuana, another lipophilic drug. This compound is detected in the urine of users for weeks or months after use, a fact that precludes accurate time estimates of last use

(Baselt 1982, Hawks 1986, Houts, et al. 1988). For those benzodiazepines that are quickly eliminated, such as alprazolam and oxazepam, it seems reasonable to assume that the urine would test negative seven days post dose (Dawson, et al. 1984, Greenblatt 1981). It should be emphasized however that for both the short and long half life metabolites, the long term detectablility in urine following chronic use has not been determined experimentally.

The typical positive test result will indicate the presence of oxazepam, temazepam or nordiazepam, indicating the use of diazepam, chlordiazepoxide, temazepam or many of the other related benzodiazepines, separately or in combination. Patterns and concentrations of certain metabolites may, however, be highly suggestive of the parent drug ingested. Temazepam, which is a minor metabolite of diazepam and is prescribed as a sedative, is eliminated in the urine almost entirely unchanged. The urine of the temazepam user has relatively high levels of temazepam, none of the nordiazepam and very little of the oxazepam seen after diazepam use. The presence of very high levels of temazepam and the absence of other metabolites makes temazepam use highly probable.

Many other specimens will not lend themselves so easily to interpretation because of other metabolites present or because the relative amount of temazepam is low. This may be the case in diazepam use, or possibly in temazepam use if the specimens was collected during the late elimination phase. The relative concentrations of metabolites for many drugs in urine change dramatically from the early to late elimination phase. To what extent this occurs for temazepam and other

compounds of the class is unknown. For this reason it is generally unsafe to predict the drug ingested from metabolites shared by many drugs.

Urinary excretion profiles for abused drugs are seen with increasing frequency in the literature primarily to aid in the interpretation of test results and in the design of procedures (Cone, et al. 1989a, 1989b, Joern, et al. 1987, Verebey, et al. 1982). Before new drugs are approved for sale, studies are routinely performed to show the relative percentages of metabolites eliminated in the urine. Usually, the percentages reflect either a single time point post dose, or a fraction of the total eliminated (Eberts, et al. 1980, Schwarz 1979). Although useful, more recent work is designed to more directly address issues of drug testing. Flurazepam for example is confirmed in some laboratories by the identification of HO-ethylflurazepam and in others by the identification of desalkylflurazepam. The advantage in terms of detectability, if any, in the identification of one compound over the other is unknown.

The compounds included in the assay were those that are frequently prescribed (Simonsen 1987). With the exception of lorazepam, all react well with RIA. Whether or not these prescription trends will be reflected in urine test results will require the analysis of a large specimens population over a long period of time. The small number of specimens tested does not allow this sort of association to be made. It is nevertheless somewhat surprising that results for the limited number of specimens tested yielded few positives for some frequently prescribed drugs. Alprazolam, which is frequently prescribed, is infrequently

detected. The vast majority of positive results were for oxazepam, nordiazepam, temazepam or flurazepam. This may be because illicit use of diazepam is high and not accounted for in surveys or because alprazolam use is restricted to a population not subject to drug testing. Another more likely explanation is that those compounds with long half lives will be the same compounds most likely to be detected. Alprazolam has a relatively short half life and so the urine of a user will be positive for only a short period of time.

It is important to note also that only those compounds that are detected by RIA will be confirmed by GC/MS. To a good first estimate, all benzodiazepines react with the assay described, but cross reactivities differ substantially. Both lorazepam and triazolam have low cross reactivities with Roche RIA. It is likely then that these drugs will only be detected in combination with other benzodiazepines capable of triggering an RIA positive. Lorazepam may be detectable using other immunochemical tests and was included in the GC/MS assay for this reason.

In summary, the difficulties encountered in testing for this class of compounds are related to the multiplicity of compounds, the chemical nature of the compounds tested, and especially the chemical diversity within this class of compounds. For this reason, finding a common derivatization scheme was central to the work. It would appear that TBDMS derivatives are the most suitable for use in a comprehensive analysis of benzo- diazepine metabolites since they are stable, react with the compounds of interest and allow for a high degree of precision.

APPENDIX A. Cross reactivity of Benzodiazepines with Roche Abuscreen Radioimmunoassay. Adapted from Abuscreen package insert (1989).

Benzodiazepine/ Metabolite(s)	Approximate ng/mL equivalent to 100 ng Oxazepam/mL	Approximate Percent Cross-Reactivity
Alpha-hydroxyalprazolam	76	131
Alprazolam	69	144
4-Hydroxyalprazolam	269	37
*Chlordiazepoxide	2,125	5
Clonazepam	5,356	2
*Clorazepate	451	22
Demoxepam	385	26
Desalkyflurazepam	442	23
Desmethylchlor-diazepoxide	336	30
Desmethylflunitra-zepam	971	10
Desmethylmedazepam	3,400	3
*Diazepam	40	284
Didesethylflurazepam	536	19
Flunitrazepam	320	31
Flurazepam	4,105	2
*Halazepam	404	25
Hydroxyethylflura-zepam	337	30
Lorazepam	3,214	3
*Medazepam	335	30
Midazolam	182	55
N-Desmethyldiazepam	49	202
N-Methyloxazepam (Temazepam)	47	212
Nitrazepam	320	31
*Pinazepam	76	131
*Prazepam	472	21
3-Hydroxyflunitrazepam	474	21
7-Acetamidonitrazepam	>10,000	<1
7-Aminonitrazepam	>10,000	<1
Triazolam	504	20
Alpha-hydroxytriazolam	2,011	5
4-hydroxytriazolam	799	13

*Benzodiazepines metabolizing to oxazepam.

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