Review Article

A Critical Review of Gas Chromatography – Mass Spectrometric Analysis of Benzodiazepines in Matrices of Forensic Importance

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ABSTRACT

Benzodiazepines are one of the most prescribed medicaments for their therapeutic properties as anxiolytics (anti-anxiety agents), hypnotics and sedatives, anesthetics, anti-convulsants and as myorelaxants and are often encountered in toxicological cases. These drugs are often used in drug facilitated sexual assaults, kidnapping, robbery and passenger duping due to their action as CNS depressants. Even due to their high potency, even a low concentration of drug results in immediate effects. The concentrations of these compounds in samples are in micrograms which make it even more important to use techniques which are very sensitive and require very less quantity of samples. Medical and forensic importance of 1,4-benzodiazepine derivatives emphasize the need of a sensitive and reliable method for their analysis. In the present study, the analytical techniques and methods for analysis of benzodiazepines from different matrices are reviewed. The review was preceded by a short introduction and action of benzodiazepines.

Keywords: Anesthetics; Benzodiazepines; Anti-anxiety agents; Hypnotics and sedatives; Gas Chromatography Mass Spectrometry; Solid Phase Microextraction.

INTRODUCTION

1.1 Background

Benzodiazepines are a class of CNS (Central Nervous System) depressant drugs, that are used for the treatment of anxiety disorders, panic disorders etc. In addition to anxiolytics (for relief from anxiety), these are also used as hypnotics (for producing sleep), anesthetics (for amnesia i.e. for impairing short term memory), anti-convulsants (stops fits and convulsions) and myorelaxants (or muscle relaxants).[1,2] The term benzodiazepine refers to the portion of the structure composed of a benzene ring (A) fused to a seven membered diazepine ring (B). Benzodiazepines means 5-aryl-1, 4-benzodiazepines, as all the important benzodiazepines contain 5-aryl substituent (C) and a 1,4-diazepine ring (i.e. nitrogen at position 1 and 4), 5-aryl-1, 4-benzodiazepines basic structure.[2] A lot of methods are available for the analysis of benzodiazepines from both the pharmaceutical perspective and forensic perspective. Since the benzodiazepines are one of the most abused drug this review has been conducted as an attempt to compare the available analytical techniques and methods for the analysis and detection of benzodiazepines in various matrices. For this purpose the literature was searched from Google scholar, Sciencedirect and Pubmed. These research papers were then critically analyzed and then this review was shaped.

1.2 History of Benzodiazepines

In 1955, Leo Sternbech, chemist by profession, discovered accidently the first benzodiazepine viz. chlordiazepoxide. Later on in 1960, it was marketed as Librium by Hoffmann-La-Roche, who also marketed another benzodiazepine Diazepam (under the trade name od Valium). After that benzodiazepines were widely available for medical purposes and in mid to late 1970s, benzodiazepines topped all 'most frequently prescribed' lists.[6] Any substance, the possesion or supply of which is restricted by law because of its potential harmful effects on the user is known as controlled or schedulded substance.[3] According to UN Convention on Psychotropic Substance (1971), 34 benzodiazepines are categorized/ listed under Schedule IV drugs, indicating that there are legitimate medical indication for their use with moderate to low abuse liability potential.[1,4] Among

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34 benzodiazepines listed in Schedule IV of UN 1971 Convention, those used as anxiolytics are alprazolam, chlordiazepoxide, clonazepam, clorazepate, diazepam, lorazepam and oxazepam, those used as hypnotics are estazolam, flurazepam, quazepam, temazepam and triazolam, those used for amnesia (for anasthesia) are chlordiazepoxide, diazepam lorazepam, midazolam, those used as anti-convulsants are clonazepam, chlorazepate, diazepam, those used as myorelaxants include diazepam.[2] Flunitrazepam, is one of the benzodiazepine which is often associated with date-rape drugs or DFSA (Drug Facilitated Sexual Assault). That is why flunitrazepam was moved to Schedule III of UN 1971 Convention.[3] According to the International Narcotics Control Board, in the last ten years the most commonly used benzodiazepine were alprazolam, chlordiazepoxide, diazepam, flunitrazepam, lorazepam, lormetazepam, nitrazepam, temazepam, and triazolam.[5]

1.3 Classification of Benzodiazepines

Based upon their half-life elimination period, benzodiazepines can be divied into four categories:

a) **Ultra short acting benzodiazepine,** whose t1/2 < 5 hours and are non-accumulating agents e.g. midazolam

b) **Short acting benzodiazepines,** whose t1/2 <6 hours and are non-accumulating agents e.g. triazolam

c) **Intermediate acting benzodiazepines,** whose t1/2 is (6-24) hours and the compounds generated by their metabolites are generally non-active e.g. estazolam, temazepam

d) **Long acting benzodiazepines,** whose t1/2 >24 hours and they generate long half-life active metabolites e.g. flurazepam, diazepam, quazepam, clobazam, chlorazepate. With repeated administration, they accumulate extensively in the body.[2,7]

Benzodiazepines having longer half-life period are typically prescribed for the treatment of anxiety disorders e.g. diazepam and lorazepam, whereas those with shorter half-life period are used as hypnotics for insomnia e.g. triazolam. [8] Benzodiazepines are metabolized through N-dealkylation, hydroxylation and conjugation pathways by hepatic enzyme CYP (Cytochrome P450). Some benzodiazepines like oxazepam are not metabolised by these enzymes, instead they are directly conjugated.[2,3]

1.4. Pharmacological Action

The main target of benzodiazepines is GABA (Gamma Amino Butyric Acid) receptors in the brain. GABA is an amino acid present in CNS in the cerebral cortex, and acts as an inhibitory neurotransmitter i.e. it inhibits nerve transmission in the brain thus blocking brain signals, calming nervous activity.[2,3] GABA receptors have three main types: A (ligand gated chloride ion channel), B (protein coupled receptors) and C (transmitter gated chloride channel). Benzodiazepines act at GABA-A receptors. They do not stimulate it directly. Instead, they make it more efficient by increasing the frequency with which the chlorine channel opens with when GABA binds to its own site on this receptor and causes hyperpolarization of membrane leading to calmness, sedation and other symptoms.[2,3] Therapeutic index of benzodiazepines is quite high (i.e. a large amount of its therapeutic dose is required to produce lethal effects), making them much safer than barbiturates, thus they easily replaced them.[2]

1.5. Addiction, Dependence and Tolerance

Benzodiazepines can develop physical and psychological dependence, within a few weeks or months of regular or repeated use. Persons taking benzodiazepines find difficulty in stopping the drug or reducing the dose because of withdrawl symptoms.[9] Dependence risk increases with dose and treatment duration and the latter should not exceed 4 weeks for insomnia and 8-12 weeks for anxiety. Beyond this time period, use of benzodiazepines increases the risk of dependence. It has also been found that benzodiazepines have been found little effective in the treatment of anxiety after four months's continuous treatment.[10] With regular use of benzodiazepines, a patient can develop tolerance, i.e. the patient feels less effect with the original dose of the drug and a higher dose is required to obtain the original effect. Although, tolerance can develop at variable rates and to different degrees.[3,9,10] Often, the persons or dependents maintain their drug supply by persuading their doctors to escalate the size of prescriptions or buy them illicitly.[3] Such user may take the path of forged prescriptions, theft from drug stores or illegal imports to fulfil their craving.[9] Tolerance to anxiolytic effects seems to develop more slowly than dose tolerance to the hynotic effects. Dosage escalation often maintains the cycle of tolerance and dependence. As a result, the patients may feel difficulty discontinuing the therapy.[10] Withdrawl symptoms can occur upon discontinuation

of long term use of benzodiazepines. Doses that are higher than usual therapeutic dose show more withdrawl symptoms than therapeutic dose. Withdrawal symptoms occur more quickly and more intensely for benzodiazepines having short half-life period than for benzodiazepines having long half-life period. These can be in the form of anxiety, insomnia, automatic hyperactivity, depression, agitation, confusion, diplopia, gastro-intestinal distress, palpitations, etc.[7] Any drug, that because of some desirable effects is used for some purposes other than that intended is known as drug of abuse and benzodiazepines are one of the drugs that fall under the category of drugs of abuse.[3] Benzodiazepines abuse may be in two forms, firstly, persistent therapeutic use, that is, use longer than generally recommended; and secondly, illicit or recreational use, in which the drug is self-administered without physician approval or supervision. The former type of abuse is common and typically involves use at low doses compared to the rarely encountered illicit use that may involve high doses and clear indications of acute intoxication and impairment.[8] Benzodiazepines are used illicitly or recreationally to increase the kick obtained from illicit drugs particularly opiates, to alleviate the withdrawal symptoms of other drugs of abuse (opiates, barbiturates, cocaine, amphetamine and alcohol) and to obtain 'high'.[9] Benzodiazepines are also used as an adjunct treatment in anti-alcoholic therapy and sometimes, people become dependent upon benzodiazepines and may abuse illicitly obtained benzodiazepines. High doses of benzodiazepines are used occasionally to obtain 'high'. Although recreational users of benzodiazepines are relatively small, but there are large number of long term therapeutic dose users of benzodiazepines.[3,9] It is estimated that every year about 10–20% of adults living in the developing countries take these drugs.[5,8] According to DAWN report (Drug Abuse Warning Network) 2014, by SAMHSA (Substance Abuse and Mental Health Services Administration), estimated number of Emergency Department visits involving benzodiazepines was increased from 46,966 in 2005 to 89,310 in 2011. Emergency department visits involving benzodiazepines in combination with other drugs like opioids, alcohol has also been increased to a significant numbers from (2005-2011) and in this data number of patients of age (12-34years) were the highest followed by patients of age (45-64years), (35-44years) and (65years or older).[11] A number of death cases involving benzodiazepines have also been reported.[12]

2. Analytical Status

Due to their widespread use and abuse, benzodiazepines are among the most commonly encountered prescribed drugs in forensic analysis.[8] So, for their detection, identification and quantitation from biological (body fluids and viscera) and non-biological samples (pills, tablets, powders, etc.), methods are needed that should be sensitive, specific, accurate and reproducible. A lot of research has already been done upon the analysis of benzodiazepines. Techniques like immunoassay, HPLC (High Performance Liquid Chromatography) or GC-ECD (Gas Chromatography- Electron Capture Detector) are used by many of the forensic laboratories for the screening of benzodiazepines, but sensitivity, specificity and precision or any combination of the three is often lacking. In those cases, capillary gas chromatography with mass spectrometric detection is the method of choice for confirmation and quantitation of benzodiazepines due to its rapidity, reliability, specificity and sensitivity.[13]

2.1. Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS is a hyphenated instrumental technique, in which GC separates out the organic volatile and semi-volatile compounds with great resolution and the separated components are detected and identified by MS on the basis of their respective charge to mass ratio. This technique proves a very important tool in analytical chemistry because it can be used for both identification and quantification of volatile and semi-volatile organic compounds in a mixture. In GC, the components of mixture in a gaseous state are separated as the sample passes over a stationary liquid or solid phase. If the stationary phase used is solid, technique is referred as Gas Solid Chromatography (GSC) and if the stationary phase used is a liquid, it is termed as Gas Liquid Chromatography (GLC). Out of these two, GLC is by far the most common. GC is based upon the principle of partition, i.e. the components of the vaporized samples are separated due to relative differences of dissolution and partition into different phases/ layers. Table no 1 illustrates different instrumental parameters used by different authors for the analysis.

2.2. Selective applications of GC – MS and LC - MS for analysis of 1, 4-benzodiazepines from various matrices

Analytical methods like GC-MS, LC-MS, GC, FTIR etc.

are routinely used for the analysis of benzodiazepines in forensic laboratories. Chromatographic techniques like GC and HPLC etc. allow separation of complex mixtures of benzodiazepines and spectroscopic techniques like MS, when attached to them, enable us identification of individual benzodiazepines in biological material blood, urine, hair etc. as done by different researchers in the following way:

2.2.1. Blood and Serum

Blood is a bodily fluid that circulates through arteries and veins, supplying the tissues with oxygen, and taking away carbon dioxide to be excreted comprising red blood cells, white blood cells, and platelets, floating in straw-colored liquid called plasma and is used as one of the biological matrix for the detection of drugs like benzodiazepines. Cairns et al.[13] analyzed alprazolam and triazolam, from hemolysed blood and liver digest samples by GC/MS/NICI. Based upon limit of quantification, it was observed that alprazolam (LOQ = 4µg/l) was less sensitive than triazolam (LOQ = 0.5µg/l) when analyzed by NICI. LOQ for alprazolam and triazolam may be reduced by using lower range of calibration standards and by extracting and reconstituting the sample in a lower volume of solvent. Calibration curves could not be differentiated for hemolysed whole blood and for liver digest. Water could be used as a matrix for calibration standards, but sensitivity is lowered. Thus spiked blood samples are recommended as calibration standard for both blood and tissues digest. Systematic loss of analyte was observed in both blood and liver digests when stored overnight at 4°C or frozen in silanized glass bottles. But, samples can be recovered by doing ultrasonication (applying ultrasonic frequency to agitate the particles in a sample) of samples for 5 minutes before removing aliquots for analysis. Adsorption of alprazolam and triazolam in trace levels was also observed onto silanized glass surface even in the presence of lipid and blood protein. Alprazolam and triazolam recoveries were found 90% better when vortex extracted once with n-butyl chloride in the presence of urea. Thus GC-MS came out to be a sensitive and specific method for analyzing alprazolam and triazolam and other benzodiazepines in clinical and postmortem samples. Cirimele et al.[14] analyzed samples of plasma, hair and sweat of schizophrenic patients for the determination of clozapine by GC-MS. No correlation was observed between daily dosage and clozapine concentration in plasma, but significant correlation was observed between daily dosage and clozapine concentration in hair. However, individual correlation was not observed due to inter-individual variations. Inspite of all this, hair was found as unsuitable specimen for monitoring exposure on weekly basis while sweat as specimen was found more effective than plasma and hair for obtaining a cumulative estimate of drug exposure over several hours (24 hours) because significant correlation was observed between daily dosage and clozapine concentration in sweat with high inter-individual variations but lower in comparison with plasma and hair data. Sweat appeared to be better matrix than plasma and hair testing for therapeutic clozapine monitoring in schizophrenic patients for qualitative analysis rather than quantitative analysis. The given method is simple, specific and do not require a derivatization step before injection of extracts and this new technique is complementary of urine and hair investigation. Inoue et al.[15] analyzed whole blood samples for the determination of benzodiazepines, using Solid Phase Extraction and GC-MS. Except oxazolam and cloxazolam, nineteen benzodiazepines and two thienodiazepines were well separated on their SIM (Selected Ion Monitoring) and TIC (Total Ion Chromatogram) chromatograms. Reproducibility of retention time was found excellent with coefficient of variation less than 0.03%. Thus the method used is simple and sensitive for the determination of benzodiazepines in whole blood and is very useful in practice of forensic science. Rasanen et al.[16] analyzed 506 urine and blood samples by gas chromatography and immunoassay techniques for the detection of benzodiazepines. Delayed retention time and tailing of peaks was observed when using acetic anhydride for acylation. Detection of 7-aminonitrazepam was not possible with the EC technique due to the lack of an electron withdrawing group. They observed that numbers of positives were highest when analyzed by GC (200), higher when urine samples were hydrolyzed before immunoassay (175) and low when urine samples were analyzed intact i.e. unhydrolysed (153). Enzymatic hydrolysis improved the detection limits in immunoassay techniques for compounds forming glucuronide conjugates. Given GC method for blood found to be a good alternative to the common combination of urine immunoassay followed by quantitative analysis of blood by chromatography, in spite of the limitations for detecting amino metabolites. Thus gas chromatography serves a very good technique for detection of benzodiazepines from blood or urine samples, inspite of the limitation for detecting amino metabolites. Synder et al.[17] analyzed urine and serum samples for the

detection of flunitrazepam and its metabolites after a single oral dose by immunoassay and GC-MS. In case of serum, SBENZ assay (Serum Benzodiazepine) gave better sensitivity than TDX assay for the detection of the presence of flunitrazepam and its metabolites, due to the lower LOD and the higher cross-reactivity to the major metabolite of flunitrazepam, i.e. 7-aminoflunitrazepam of SBENZ assay. Also, with acid hydrolysis GC-MS gave much higher values and the relative sensitivities of assays for the detection of flunitrazepam ingestion came out to be as: GC-MS > SBENZ > TDX for both urine and serum samples i.e. GC-MS is the best method. Aebi et al.[18] analyzed human blood samples for the analysis of bromazepam, diazepam and nordiazepam qualitatively and quantitatively using GC-TOF-MS. They observed that TOF system yielded high quality spectra, despite the low amount of three compounds. Due to high level of background signals, the presence or absence of bromazepam could not be established with GC-ECD and HPLC-DAD. GC-TOF gave better sensitivity over quadrople GC-MS in qualitative analysis and it proved better from the two techniques in quantitative analysis. They concluded that GC-TOF-MS has the ability of giving effective and robust analysis of sample with strong matrix interference. Pirnay et al.[19] used GC with Ion Trap Tandem MS for the detection of 22 benzodiazepines in human blood and urine samples. They observed that the trimethylsilylation of benzodiazepines including a protic functional group increases their MS–MS detection thresholds by a factor of 10–1000. It also showed that the most sensitive ionization mode depends on the drug considered. They concluded that for analysis of whole blood and urine extracts with efficient and unambiguous detection of the selected drugs, GC-MS-MS serves a very good technique. Papoutsis et al.[20] determined benzodiazepines and their metabolites from blood samples by EI-GC-MS and also validated this method also. It was observed that the recoveries were higher than 74% for all the benzodiazepines. The calibration curves were linear within the dynamic range of each benzodiazepine with a correlation coefficient higher than 0.9981. Accuracy and precision were found to be less than 8.5% and 11.1% respectively. It was concluded that the developed method is rapid, sensitive and reliable for the simultaneous determination of 23 benzodiazepines in blood sample. This method is suitable not only for the evaluation of pharmacokinetics, bioavailability and clinical pharmacology of benzodiazepines, but also to detect and identify them and/or their metabolites in blood samples concerning the investigation of road traffic accidents, drug overdoses and suicidal or accidental poisonings taking average time for each sample analysis is less than 2 hours and making it suitable for Emergency toxicology laboratories.

2.2.2. Hair

Drugs and their metabolites are incorporated into the hair matrix from the bloodstream following drug administration. Hair drug testing detects drugs that are embedded in the hair. Also, detection time is the longest in hair that enables the detection of drug after even after 90 days of its administration. Cirimele et al.[21] analyzed human hair samples by GC/MS (MS was operated in NCI mode) for the detection and quantification of lorazepam. They observed the increasing concentrations of lorazepam from the end to the roots of a 16cm long hair strand and established that the driver had taken the drug over a long period of time. They concluded that GC-MS in NCI mode proved a very good and sensitive technique for the detection and quantification of lorazepam and other benzodiazepines in hair samples. Hold et al.[22] analyzed human hair samples for the qualitative and quantitative determination of alprazolam by GC-MS in NCI mode. It was observed that in pigmented and non-pigmented hair, alprazolam was easily detected in the levels of (60-100) pg/mg range. They concluded that GC-MS with NCI proved to be a sensitive, precise and accurate method for the qualitative and quantitative analysis of alprazolam in rat hair. Yegles et al.[23] analyzed benzodiazepines and other psychotropic drugs in human hair samples, using GC/MS. They observed that nordiazepam was the most frequently found drug in human hair samples followed by oxazepam, diazepam, lorazepam and flunitrazepam. They also observed that 40 samples that gave positive result with post-mortem blood, 37 out of them gave positive result with hair analysis. They concluded that hair analysis is complementary to classical post-mortem analysis in forensic toxicology and present method provides us very crucial information on the degree of exposure over a long time period. Yegles et al.[24] analyzed the hair samples of a corpse, who had died after an overdose of several illicit drugs including benzodiazepines, to study the effect of bleaching agents on the stability of benzodiazepines in hair by GC-MS. Nordazepam, diazepam and 7-aminoflunitrazepam were evaluated in the study. They observed that shampoo treatment did not affect the drug content present in hair

samples. Concentration of all drugs detected in bleached hair was less than that from untreated hair due to the dehydration of drugs by peroxide and bleaching had also influenced stability of entrapped benzodiazepines in hair. They concluded that drug levels can be reduced but cannot be eliminated with external agents like bleaching etc. and it is very important to consider the cosmetic history of hair sample in the interpretation of hair analysis result. Scott et al.[25] examined rat hair samples for the determination of 8 benzodiazepines by GC-MS and HPLC by injecting those benzodiazepines daily for 10 days at a concentration of 10mg/kg. They observed that method 3 i.e. acidic methanolic extraction (with methanol/ trifluroacetic acid (50/1) gave best results in extraction procedure for all the drugs taken from hair and method 7 i.e. protease enzymatic digestion method for extraction gave the worst results. Dichloromethane proved to be a very good extraction solvent with highest recoveries and ethyl acetate proved to be a very poor extraction solvent. They concluded that by selecting an appropriate extraction method and analytical method like GC-MS, benzodiazepines and their metabolites can be detected in hair, even if they are present in very low concentrations. Kintz et al.[26] analyzed urine, oral fluid and hair samples for the detection of lorazepam using LC-MS/MS technique with a special focus on drug-facilitated crimes. They observed that limits of detection came out to be 0.02, 0.05 ng/ml and 0.5 pg/mg for urine, oral fluid and hair, respectively. Despite an extraordinary sensitivity (i.e. LOD= 0.5 pg/mg), it was not possible to detect a single dose of lorazepam in hair. They concluded that urine is the best specimen out of urine, oral fluid and hair samples to document any drug-facilitated crime involving lorazepam.

2.2.3. Urine

The most widely used matrix for the detection of drugs is urine because it offers a window of detection for (1-7) days and also, metabolites of the drugs are most likely to be detected in urine because of having longer half-life then than their respective parent compound. Maurer et al.[27] analyzed urine samples for the determination of 1,4 and 1,5 - benzodiazepines by employing GC-MS. No significant changes were observed in the retention indices of compounds with temperature programming as compared to isothermal procedure. Only androsterone was the endogenous physiological substance that appeared in the mass fragmentation pattern. They concluded that GC-MS served a very good method for the differentiation of diazepam along with its metabolites and lorazepam in a short period of time. Fraser et al.[28] analyzed 36 urine samples for alprazolam and its metabolites using Abbott ADx and TDx as screening method and GC/MS as confirmation method. They observed that out of total 36 samples, 31 were positive for α-OH alprazolam by GC/MS. 2 specimens were negative by all assays, 3 specimens contained benzodiazepines other than alprazolam and 27 specimens were confirmed with 3-HMB benzophenone. They concluded that Abbott ADx and TDx are acceptable screening assays for the detection of alprazolam use, from urine samples containing >400 ng/ml of α-OH alprazolam. EMIT assay has proved to be slightly more sensitive than Abbott systems for detection of alprazolam in this study, and the positive results from both these screening methods can be confirmed by GC/MS for positive or negative identification of substance. Also, the confirmation assays for triazolobenzodiazepines (e.g. alprazolam) should ideally be directed towards the α-hydroxyl metabolites of these drugs, not the parent drug. Lillsunde et al.[29] analyzed urine sample for the detection of commonly abused drugs (including benzodiazepines) by TLC as preliminary screening method and GC-MS as confirmatory method. They observed that drugs or their metabolites, even in low concentrations, can be detected using Chem Elut Extraction Tubes. Out of their study, they concluded that for initial screening of drugs and their metabolites, TLC serves a very good technique giving characteristic colors and retardation factor values, and for specific detection of drugs and their metabolites, GC-MS serves as the most suitable method for their confirmation and identification. Joern et al.[30] analyzed urine samples for determination of low concentrations of benzodiazepines (including alprazolam and triazolam) by GC/MS with an extractive alkylation procedure. They concluded that present method can be successfully applied for the analysis of urinary triazolobenzodiazepines metabolites at approximately 10 times lower concentrations than those of previously mentioned methods while chlordiazepoxide cannot be detected by this method. West et al.[31] described GC/MS method for the detection of oxazepam, nordiazepam, desalkylflurazepam, temazepam and α-hydroxyalprazolam in urine samples. They observed that α-hydroxyalprazolam out of all compounds tested was very sensitive to the condition of injection port linear and eluted late. Peak shapes for other compounds tested (except α-HA), was good. Also, desalkylflurazepam and nor-diazepam nearly co-elute, but the spectra of two compounds do not interfere with each other. They

concluded that extraction procedure used provides satisfactory results and it extracted all five analytes with recoveries ranging from (73-83) % and GC/MS serves a very good method for the quantitative confirmation of immunoassay results.

Black et al.[32] analyzed 18 urinary samples for the detection of diazolobenzodiazepines and triazolobenzodiazepines and their metabolites by Solid Phase Extraction and Gas Chromatography- Mass spectrometry. They observed that optimum pH for extraction is between (4-5). It was also observed that sensitivity could be increased using a splitless injector mode. This assay has the advantage like its specificity, ability to detect clonazepam metabolite in addition to other benzodiazepine metabolites and the efficiency of solid-phase extraction. Meatherall et al.[33] analyzed urine samples using GC-MS for the detection of benzodiazepine metabolites. They observed that except 2-hydroxyethylflurazepam and N-desalkyl-3 -hydroxyflurazepam, targeted benzodiazepines were well separated from each other. Due to propylation and propionylation, benzodiazepines were well identified and quantified. Needleman et al.[34] identified parent benzodiazepines from their metabolites by analyzing urine samples using GC-MS. They observed that use of α-glucuronidase to provide the free drug from its glucuronide parent compound obviates the need for acid hydrolysis. This, in turn, prevents conversion of the drug metabolites to either of the base benzophenones, with the resulting loss of unique identity of the benzodiazepine used. They concluded that excellent quantitation can be achieved by using temperature program and a splitless mode of injection within an 8 minutes run-time and urinary concentrations of the benzodiazepines > 200 ng/ml are most likely due to abuse rather than to a prescribed ingestion under strict medical surveillance. Valentine et al.[35] analyzed 924 urine samples for the detection and identification of benzodiazepine and their metabolites by HPLC and GC-MS after immunoassay screening as preliminary method for analysis. They observed that with EMIT, 788 specimens were found positive with one or more benzodiazepines or their metabolites and 136 were found to be negative. 56 samples were found negative and 55 samples were found positive for one or more benzodiazepines or their metabolites out of 136 negative samples with GC-MS. They concluded that lorazepam when present in urine in amounts greater than 1000ng/ml, will be detected by EMIT. Also presence of deoxepam in urine is a strong indicative of intake of chlordiazepoxide because deoxepam can only be found as a result of chlordiazepoxide metabolism and deoxepam was only detected by REMEDi and not by GC-MS analysis because deoxepam gets pyrolyzed to nordiazepam, which is a common intermediate in the metabolic pathway of many different 1,4- benzodiazepines. It was also found that prazepam and chlorazepate were not widely used as like other 1,4- benzodiazepines. 7-aminoflunitrazepam was also detected in urine by REMEDi and also confirmed by GC-MS. Beck et al.[36] described an online screening technique for urinary benzodiazepines and its comparison with EMIT, FPIA, and GC-MS from 50 urine samples. They observed that enzymatic hydrolysis increased the ability of the online system to detect samples containing benzodiazepines or their metabolites. Different systems showed high degree of detectability for all groups of compounds at concentration greater than 300ng/ml. Cut-off limit comes out to be 100ng/ml, which is still applied in screening assay. Conclusion drawn out of this study is that detectability can be increased with hydrolysis and a reliable screening of urine samples can be obtained by using automated enzyme hydrolysis with increased detectability. El Sohly et al.[37] analyzed urine samples for the determination of flunitrazepam and its metabolites by GC-MS. They observed that acid hydrolysis increased the sensitivity of benzodiazepines detection. Heptafluorobutyrate derivative proved to be the most useful derivative which provided the highest sensitivity and the cleanest chromatograms. It was also observed that it was necessary to carry out the derivatization process at room temperature. This method is so sensitive that it can detect 7-amino-FN and 7-amino-nor-FN, even when their concentration is as low as 1ng/ml. Thus GC-MS is a sensitive procedure for the confirmation of presence of FN metabolites in urine. Ausburger et al.[38] compared different immunoassays and GC-MS screening of benzodiazepines in 53 urine samples. They observed that sensitivity varied from 36, 64 to 75% for COBAS online, RIA immunalysis and RIA DPC respectively. In cases where lorazepam and lormetazepam were found negative with immunoassays, GC-MS gave their positive presence in all that cases. They concluded that sensitivity of drugs related to oxazepam (e.g. flurazepam or lorazepam) can be increased using hydrolysis prior to immunoassay, but it is not so for other drugs. RIA and Online techniques provide us good repeatability and specificity for benzodiazepines detection in urine but do not provide required sensitivity, particularly for lorazepam, lormetazepam and low concentrations of

benzodiazepines. For detection of therapeutic doses of benzodiazepines from urine samples, immunoassay techniques may be unreliable but GC-MS serves as the most reliable technique. Elsohly et al.[39] conducted a study to assess the prevalence of drug use in sexual assault cases from 1179 urine samples with GC-MS. They observed that LOD (limit of detection) for benzodiazepines with GC-MS assay came out to be 5ng/ml, except for alprazolam, triazolam and α-hydroxyalprazolam that LOD came out to be 50ng/ml. Additional 50 samples that were identified positive for benzodiazepines by immunoassays, were found negative by GC-MS confirmation. Conclusion drawn out of this study is that the presence of these substances in urine of individual does not necessarily indicate the involvement of these drugs in sexual incident and may reflect an exposure to the drug before or after the incident. A wide range of drugs could have been involved in cases of sexual assault. With current technology, most of these drugs can be detected within 24hours period of incident.

Guan et al.[40] analyzed urine samples for the detection of benzodiazepines using Solid Phase Microextraction and GC-ECD of their respective benzophenones. They observed that recoveries of benzophenones by SPME were generally much lower than those by ether extraction but the chromatographic responses of SPME separated analytes were higher and their detection limits were much lower than those by ether extraction. They concluded that benzophenones can be efficiently separated from hydrolyzed urine by DI-SPME and sensitively detected by GC-ECD and thus it is the recommended method for identification of benzodiazepines in clinical and analytical toxicology. Blachut et al [41] described about the applications of gas chromatography/ mass spectroscopy (GC/MS) for the analysis of benzodiazepines from human urine samples. It was observed that partial decomposition of temazepam, nitrazepam, clonazepam and oxazepam lead to obtaining chromatograms containing peaks of primary compounds and peaks of products of decomposition. Decomposition or regrouping of chlordiazepoxide, lorazepam and clorazepatic acid leads to a chromatographically stable product and active functional groups in the structure of an analyte could cause irreversible adsorption on the stationary phase of a column and hence can cause tailing. It was concluded that developed conditions enabled for detection and identification of 18 benzodiazepines present in urine samples. Hegstad et al.[42] analyzed urine samples for the determination of benzodiazepines using Solid-Phase Extraction and High Performance Liquid Chromatography-Electro spray Ionization Tandem Mass Spectroscopy. It was observed that the average recovery of different analytes ranged from 56% to 83%. LOQ (limit of quantification) was found to be between (0.02-0.01) μM. They concluded that the method used is robust and specific for identification and quantification of benzodiazepines from urine samples. Method has been validated in regards to separation, recovery, linearity and specificity. Kinani et al.[43] analyzed urine samples for the detection of diazepam and its metabolites nordiazepam and oxazepam by GC-MS-MS. They observed and concluded that extraction using Toxi-Tubes yields good recovery within a short period of time and xenon gave more satisfying CID spectra (Collision Induced Dissociation) as collision gas instead of argon and dissociation mechanism of negatively charged benzodiazepines are suggested for first time. Arnhard et al.[44] developed and validated a rapid and sensitive method for the screening and quantification of 35 benzodiazepines in human urine by gas chromatography/time-of-flight mass spectrometry (GC-TOF-MS). They observed that butyl acetate (i.e. LLE) was found better than ethyl acetate and dichloromethane/2-propanol (i.e. SPE) for elution of the benzodiazepine in case of extraction. Reproducibility was observed with coefficients of variation below 2% at concentrations of 50 and 200ng/ml. It is concluded from the study that the method developed has the ability to detect and quantify so many targeted benzodiazepines with high speed and sensitivity, with a run time of 9.5 min and an LOQ of 10 ng/ml per ml of urine. Karampela et al.[45] developed and validated a simple and rapid LC/MS method for the identification and quantification of selected benzodiazepines from urine samples. They observed that LOD and LOQ levels of all benzodiazepines tested were calculated to range between 10.27–15.61 and 31.13–47.30 ng/ml, respectively. The recoveries were found to be higher than 92% for all the benzodiazepines. Conclusion drawn out of this study is that the proposed study lacks an extraction step, as diluted urine samples were directly injected into the LC/MS system, which is the main advantage of this study. Also, the proposed method is suitable for the replacement of immunoassay screening methods, since it provides higher sensitivity and specificity. Terada et al.[46] analyzed quazepam and its metabolites in human urine samples by gas chromatography–mass spectrometry (GC-MS). They observed that the calibration curves of quazepam

and its metabolites in urine showed good linearity in the concentration range of 2.5–500 ng/0.2 ml of urine with average recoveries 71-83% and 88–90% respectively. LOD for quazepam, 2-oxoquazepam and 3-hydroxy-2-quazepam came out to be 0.096–0.37 ng/ml. It is concluded that the developed method is a sensitive method for the simultaneous quantification of quazepam (even in low concentrations) and its metabolites in human urine using an Rtx-5MS capillary column. Gahobadi et al.[47] determined ultra-trace amounts of benzodiazepines in ultra-pure water, tap water, fruit juices, and urine samples SPE coupled with dispersive liquid–liquid microextraction followed by GC-FID. They observed that the relative recoveries of the analytes from all the samples came out to be in the range of 92.5–110%, indicating that the studied real matrices had almost no effect on the extraction efficiencies. Conclusion drawn out of the study is that the proposed method is fast and simple, and characterized with ultra-PFs, low LODs, relatively wide LDRs, and short analysis times and possesses a great potential for the analysis of ultra trace compounds in water, fruit juice, and urine samples.

2.2.4. Drinks

Chen et al.[48] determined trace benzodiazepines from drinks by using direct electro spray probe/ mass spectrometry (DEP/ MS). They observed and concluded that DEP/MS came out to be an effective method for the determination of benzodiazepines from drinks. Sample analysis can be completed in less than 5 minutes including sample pre-treatment.

2.2.5. Oral fluid

Oral fluid as a matrix for the detection of drugs is used now a days as an alternative to urine and hair samples because of ease of sample collection and non-invasiveness and most important parent drugs are often found in oral fluid unlike urine, in which only metabolites of the drug consumed are found. The window of detection for oral fluid is from (5-48) hours. Langel et al.[49] analyzed 4183 oral fluid samples qualitatively and quantitatively for drugs of abuse including medicinal drugs using GC-MS. In results it was observed that all compounds except norbupreophine and phenazepam, gave linear calibration curves. Except norbupreophine (0.988) and midazolam (0.986), all analytes gave coefficient of detection>0.990. extraction efficiency of benzodiazepines ranged from 81% (nitrazepam and nordiazepam) to 120% (lorazepam). 7.2% of the samples were found positive for one or more substances analyzed. Conclusion drawn out of this study is that the developed method is the most comprehensive validated method for quantitative analysis of drugs of abuse including benzodiazepines from oral fluid samples with high throughput of sample and is sensitive for compounds even lower LOQs than the cut-offs required.

2.2.6. Review articles

Simpson et al.[50] gave a review article about the screening of different drugs of abuse including benzodiazepines from urine samples. In case of liquid-liquid extraction (LLE), extraction is best performed at pH 12 due to the weak basic nature of most of benzodiazepines with extractants ranging from benzene, benzene/ isopentanol or benzene isoamyl alcohol, benzene/ dichloromethane to tert-butyl alcohol, butyl acetate and dichloromethane or hexane/ dichloromethane (70:30). Benzodiazepines can be extracted efficiently from serum using hexane/ethyl acetate. Now days, Toxi-Lab extraction tubes are also used for extraction purpose in which Toxi-Tubes A are used for alkaline pH and Toxi-Tubes B are used for acidic pH. In case of solid phase extraction (SPE), for elution of drugs, efficient solvent for extraction found is hexane: isopropanol (9:1), in C-18 columns. C-2 and C-8 columns are also found efficient for extraction. Conversion of benzodiazepines to their benzophenones has also been discussed and for unstable parent benzodiazepines and is carried out by treating the sample with HCl at 100°C. Derivatization during extraction was also found efficient and was done by adjusting the pH to 13 using 0.1M KOH and extracting with an ion-pair like tetrahexyl ammonium hydroxide (0.01M) in the presence of methyl iodide. Then finally confirmation of the positives can be done using instrumental techniques like TLC, GC-MS, HPLC, LC-MS etc. In case of GC-MS, confirmed procedure is enzymatic hydrolysis of the sample followed by extraction followed by derivatization and final analysis by GC-MS. The cut-off level for benzodiazepines has been set at (200-300) µg/L for the parent drugs. It should be noted that whatever method is used for the analysis of benzodiazepines or their metabolites, the imprecision at the cut-off should be known and a procedure adopted for the reporting of results whether positive or negative, fall within 2SD of the cut-off. Uddin et al. have reviewed different analytical techniques like chromatography (HPLC, TLC, GC), capillary electro-chromatography, capillary electrophoresis, immunoassays, photometric and electroanalytical techniques for the analysis of 1,4- benzodiazepines regarding their sample pre-

treatment, extraction for isolation of benzodiazepines from complex bio-matrices. For extraction procedure, LLE i.e. liquid-liquid extraction (44%) and SPE i.e. solid-phase extraction (53%) are widely used out of a number of extraction techniques like Micro-extraction (SPME, LPME)—6%, Column Switching (CS)—4%, Dialysis—4% etc. TLC has shown little interest in the scientific publications over the last two decades because of lack of sensitivity and specificity, particularly for the newer more potent benzodiazepines. Capillary electrophoresis (CE) has emerged as a new powerful method for rapid separations of analytes with very promising and interesting application of microscale analysis of drugs. On the other hand, having advantages of convenience and speed, spectrophotometry, fluorimetry or polarography lacks the sensitivity and specificity, requiring large amounts of sample and also unable for the simultaneous determination of benzodiazepines. HPLC offers an attractive analytical alternative for the routine determination of 1,4-benzodiazepines and its metabolites in biological samples having ability to achieve detection limits for many benzodiazepines using UV or DAD detection down to 1–2 ng Ml-1 with 1–2 ml of urine or serum. In HPLC, ECD detectors give better detection limits than NPD detectors. Gas chromatographic (GC) methods offer excellent sensitivity, but possess some drawbacks over HPLC like lengthy clean-up procedures, formation of more volatile derivatives or hydrolysis prior to analysis, decomposition of certain benzodiazepines due to high temperature of column. Conclusion drawn out of the study is that LC–MS was proved to be the most useful tool in sensitivity terms for identification of ng/ mg levels of benzodiazepines in human hair. Chromatographic techniques, particularly HPLC and GC, are most commonly used to identify specific benzodiazepines present in a sample, sometimes initially screened by one of the immunoassay-based kit methods. Persona et al.[5] gave a review article of analytical methods that are used for the determination of benzodiazepines from biological samples. The study advocates the necessity of searching for new biological matrices suitable for the detection and quantification of benzodiazepines. They also observed the potential utility of two non-typical samples (for this kind of analysis) i.e. fingerprint deposits and exhaled breath. They concluded that analytical methods like LC with MS (or MS/MS), HPLC-DAD (with UV detection) and GC withdetection methods MS, ECD or NPD play a dominant role for the determination of benzodiazepines in biological samples.

DISCUSSION

Benzodiazepines are the drugs that act upon CNS and are commonly prescribed by doctors as sedatives, anxiolytics, muscle - relaxants and for panic disorders also. Because of their immense applications and their potential for abuse, these drugs are encountered very frequently in clinical toxicology and in forensic toxicology respectively. Hydrolysis (acid or enzymatic) of the encountered samples is the foremost step and necessary condition to provide the free drug from its glucuronide parent compound followed by screening with Immunoassay techniques. Derivatization of the screened samples can also be done (using BSTFA (N,O-bis(trimethylsilyl)-trifluoroacetamide) and TMCS (trimethylchlorosilane) or TMS (Trimethylsilane) or TMCA), but not necessary. Authors have also suggested the use of derivatization agents such as acetic anhydride and protic functional groups to increase the sensitivity. The drawback with acetic anhydride is that it causes the tailing in mass spectra peaks but the protic functional groups seem to increase the MS detection threshold by a factor of 10 – 1000. LLE (Liquid Liquid Extraction) and SPE (Solid Phase Extraction) came out to be the most widely used extraction techniques out of SPME (Solid Phase Micro-extraction), MAE (Microwave Assisted Extraction), CPE (Cloud Point Extraction) etc. Extracted samples can be confirmed and quantified using a number of instrumental techniques like GC-MS, HPLC-DAD, LC-MS, GC-FID, EI-GC-MS, GC-TOF-MS, but GC-MS and LC-MS came out to be the most sensitive and specific techniques for detection and quantification of benzodiazepines. Lower LOD and LOQ have been reported using hyphenated techniques for the analysis of benzodiazepines. Hyphenated techniques also provided higher accuracy and better precision along with better recovery rates for both benzodiazepines and their metabolites. Therefore, it is accurate to say that hyphenated techniques are more suited and better choice of analytical methods not just for for the evaluation of pharmacokinetics, bioavailability and clinical pharmacology of benzodiazepines but also the analysis, detection and identification of benzodiazepines in various matrices often encountered in forensic cases.

CONCLUSION

The present review was conducted in an attempt to compare the available analytical methods and techniques for the analysis of benzodiazepines. The liquid liquid extraction and SPME were found to be most widely used

extraction method along with GC-MS and LC-MS to be most widely used analytical methods. While using any analytical method we must keep in mind the type of matrix used. More research must be done in this field in order to develop new methods for the analysis of benzodiazepines especially in cases of forensic significance. Such methods will result in better and reliable interpretation of the results and in formulating the cause of death in many poisoning cases.

CONFLICTS OF INTEREST

Declared none.

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Table No 1 – List of Various Methods Used for the Analysis of Benzodiazepines

S. No.	Analytes	Matrix	Extraction Technique	Column (Dimensions)	Carrier gas (flow rate)	Injector Temperature (0C)	m/z Range	References
1.	1,4 and 1,5-Benzodiazepines	Urine	LLE	DMCS 80-100 (60cm x 1mm i.d.)	He (7ml/min)	270°C	230-244	27
2.	Different drugs including Benzodiazepines	Urine	SPE	SE-54 (25m X 0.3 mm X 0.17 µm)	NR	280°C	57- 205	29
3.	Alprazolam and metabolites	Urine	LLE	DB-1 column (15 m x 0.26 mm i.d.)	NR	NR	NR	28
4.	Diazolo - and triazolo-Benzodiazepines and metabolites	Urine	LLE	DB-5 (30 m x 0.25 mm i.d. x 0.2µm)	He 0.9ml/min	260°C	77- 394	30
5.	Oxazepam, nordiazepam, desalkylflurazepam, temazepam, α-hydroxya-lprazolam	Urine	LLE	HP 5890 (12.5m)	NR	270°C	327- 464	31
6.	Diazolobenzodiazepines and triazoloben - zodiazepines and metabolites of lorazepam, clonazepam, alprazolam, and triazolam.	Urine	SPE	HP 5995 (25 m x 0.2 -mm i.d. x 0.33-µm)	He 0.5ml/ min	250°C	NR	32
7.	Diazepam, nordiazepam, temazepam, oxazepam, lorazepam, α-hydroxyal-prazolam, α-hydroxytriazolam, 2-hydroxyethylflurazepam, N-desalkyl-3-hydroxyflurazepam, and N-desalkylflurazepam	Urine	LLE	DB-1 (15m x 0.25-mm i.d. x 0.25)	He	135°C for 0.5mins to 285°C for 8mins (Programmable Injector was used)	57- 422	33
8.	Benzodiazepines	Urine	LLE	DB-5 (12 m x 0.25 m i.d. x 0.25 µm)	NR	255°C	205-343	34
9.	Benzodiazepines	Urine	LLE	HP 5890 (12 m x 0.2 mm i.d. x 0.33µm)	He 1.15 ml/ min	250°C	287- 436	35
10.	Flunitrazepam and its metabolites	Urine	LLE	HP 5890 (25m x 0.2 mm i.d. x 0.33µm)	He	250°C	211- 636	37
11.	Benzodiazepines	Urine	SPE	HP 6890 GC, 30m, 0.25 mm I.D., 0.25 mm	NR	NR	NR	36
12.	Benzodiazepines	Urine	LLE	HP 6890 HP-5MS (30m x 0.25 mm i.d. x 0.25 µm)	NR	NR	50- 290	38
13.	Benzodiazepines	Urine	SPME	DB-17 (30 m x 0.32mm i.d. x 0.25µm)	He	270°C	NR	40
14.	Benzodiazepines	Urine, blood	LLE (for blood), SPE (for urine)	HP-1 (12 m x 0.20 mm i.d. x 0.33 µm)	NR	250°C	272- 399	16

15.	Flunitrazepam and its metabolites	Urine, serum	LLE	DB- 5 (25m x 0.2mm i.d. x 0.33µm)	He	250°C	257- 639	17
16.	Benzodiazepines	Blood, urine	SPE using Toxi tubes	DB-5 (30m x 0.25 mm i.d. x 0.25 mm)	He 1.0ml/ min	250°C	168- 391	19
17.	Benzodiazepines	Urine	SPE	HP-5MS (30m X 0.25 mm i.d. x 0.25µm)	He 0.6ml/ min	250°C	40- 320	41
18.	Benzodiazepines	Urine	SPE and LLE both	VF-DA (12 m x 0.2 mm i.d. x 0.33 µm)	He 1ml/ min	280°C	50- 500	44
19.	Quazepam and its metabolites, 2-oxoquazepam and 3-hydroxy-2-oxoquazepam	Urine	SPE	Rtx-5 (15 m x 0.25 mm i.d. x 1.0µm)	He 2.7ml/ min	250°C	60- 480	46
20.	Diazepam, nordazepam and oxazepam	Urine	LLE	VF-5 (30m x 0.25 mm i.d. x 0.25µm)	He 1.4ml/ min	280˚C	50-400	43
21.	Benzodiazepines	Ultra-pure water, tap water, fruit juices, urine	SPE	HP-5 (30m x 0.32 mm i.d. x 0.25µm)	He 3.0ml/ min	280°C	NR	47
22.	Alprazolam and triazolam	Blood, liver	LLE	Ultra-2 (25 m x 0.2-mm i.d. x 0.33µm)	He	280°C	NR	13
23.	Benzodiazepines	Blood	SPE	BPX-5 (15m x 0.32 mm i.d. x 0.25 µm)	He 50ml/ min	280°C	242- 394	15
24.	Bromazepam, diazepam and nordiazepam	Blood	LLE	DB- 5 (17m x 0.25mm i.d. x 0.1µm)	He 4.4ml/ min	NR	50- 550	18
25.	Different Benzodiazepines	Blood	LLE	HP-5 (30m x 0.25mm i.d. x 0.25)	He 1ml/ min	300°C	72- 359	20
26.	Lorazepam	Hair	LLE	HP-5 (30 m x 0.25 mm i.d.)	He 1ml/min	250˚C	302	21
27.	Chlordiazepoxide, diazepam, estazolam, flunitrazepam, flurazepam, medazepam, oxazepam and triazolam	Hair	Seven extraction methods were used	TC-1 (20 m x 0.25 mm i.d. x 0.25 µm)	He	250˚C	80-440	25
28.	Lorazepam	Hair	LLE	HP-5 (30 m x 0.25 mm i.d.)	He 1ml/ min	250°C	100- 580	14
29.	Oxazepam, Nordiazepam, Diazepam, Flunitrazepam, Carbamazepine, Lormetazepam, Lorazepam	Hair	SPE	HP-Ultra 2 (12 m x 0.2 mm i.d. x 0.33 µm) 2ml/min	He	260°C	58- 307	23
30.	Nordiazepam, Diazepam, 7-aminoflunitrazepam	Hair	SPE	HP-Ultra 2 (12 m x 0.2 mm i.d. x 0.33 µm)	He 1 ml/min	260°C	256 - 479	24
31.	Different drugs including Benzodiazepines	Oral fluid	SPE	DB-5HT (15m x 0.32mm i.d. x 0.10µm)	He 3.5ml/ min	300°C	72- 433	49

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