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MINISTRY OF HEALTH OF UKRAINE
NATIONAL UNIVERSITY OF PHARMACY
faculty for foreign citizens' education
pharmaceutical chemistry department

QUALIFICATION WORK

on the topic: **«THE CHOICE OF OPTIMAL METHODS OF DETERMINATION OF CLONAZEPAM FOR THE TASKS OF FORENSIC PHARMACEUTICAL ANALYSIS»**

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Kharkiv – 2023 year

ANNOTATION

For the tasks of forensic pharmaceutical analysis of clonazepam, the methods liquid chromatography and ultra-performance liquid chromatography–tandem mass spectrometry are proposed, for which validation characteristics are considered, the cost of research is calculated, and environmental friendliness is determined. The qualification work consists of an introduction, three chapters, general conclusions, a list of used literary sources, laid out on 44 pages, illustrated with 6 figures, 4 tables and1 schema, containing 64 sources of literature.

Keywords: clonazepam, forensic pharmaceutical analysis, quality control methods, chromatography, green analytical chemistry.

АНОТАЦІЯ

Для завдань судово-фармацевтичного аналізу клоназепаму запропоновано методи рідинної хроматографії та надвисокоефективної рідинної хроматографії-тандемної мас-спектрометрії, для яких розглянуто валідаційні характеристики, розраховано вартість досліджень та визначено екологічність. Кваліфікаційна робота складається зі вступу, трьох розділів, загальних висновків, списку використаних літературних джерел, викладена на 44 сторінках, проілюстрована 6 рисунками, 4 таблицями та 1 схемою, містить 64 джерела літератури.

Ключові слова: клоназепам, судово-фармацевтичний аналіз, методи контролю якості, хроматографія, зелена аналітична хімія.

CONTENTS

LIST OF ABBREVIATIONS

DLLME - dispersive liquid–liquid microextraction

GAC- green analytical chemistry

GC - gas chromatography

LC - liquid chromatography

LLE - liquid–liquid extraction

LOD - limit of detection

LOQ - limit of quantitation

MS - mass spectrometry

PALME - parallel artificial liquid membrane extraction

PP - protein precipitation

RT - room temperature

SPE - solid-phase extraction

TOF - time-of-flight

UPLC–MS-MS - ultra-performance liquid chromatography–tandem mass spectrometry

INTRODUCTION

Actuality of subject. Clonazepam, a benzodiazepine, is commonly used in treating various conditions, including anxiety disorders and epileptic seizures. Due to its low price and easy availability, however, it has become a commonly misused medication, both in medical and recreational contexts. Clonazepam, alone or in combination with other psychoactive substances, can lead to unwanted effects on health, such as motor and cognitive impairment, sleep disorders, and aggravation of mood and anxiety disorders. Prolonged use of clonazepam may lead to physical dependence and tolerance.

Therefore, very often the use of clonazepam leads to addiction and leads to criminal acts. Therefore, it is often found in toxicological and forensic analysis in case files.

In this regard, it is important to select methods for the determination of clonazepam in the substance and finished medicinal products and mixtures in the presence of other components. It should be borne in mind that these methods must meet the requirements of the Pharmacopoeias for pharmaceutical analysis, the Ministry of Justice for the use of methods in forensic and toxicological analysis, so that the results obtained have legal force and serve as evidence in court.

Therefore, the methods should be validated, and their choice should be justified by the following parameters: validation characteristics suitable for court cases, environmental friendliness and cost-effectiveness.

Purpose of work is to select a method for the determination of clonazepam for pharmaceutical and forensic analysis.

The object of the research is clonazepam in substance, finished medicines and materials of court and toxicological cases.

The subject of the research is to consider the possibility of using liquid chromatography and ultra-performance liquid chromatography–tandem mass spectrometry methods for the determination of clonazepam in case files and to

compare the methods in terms of validation parameters, cost and environmental friendliness.

Tasks of work. For this objective the following tasks were supplied:

- to review the literature on the diseases for which clonazepam drugs are prescribed, their mechanism of action and pharmacological effects;
- to review statistical data on the use of clonazepam in the world, the frequency of falsification of medicines and off-label use, as well as forensic pharmaceutical analysis of case files;
- to review the physicochemical properties and modern methods of analysis of clonazepam;
- to select optimal methods for the determination of clonazepam for use in forensic pharmaceutical analysis;
- to calculate the cost of reagents, environmental friendliness and compare the validation characteristics for the proposed methods;
- to compare the results obtained and draw a conclusion about the optimal methods for the analysis of clonazepam in court cases.

Methods of the research: compilation of data from reports on clonazepam analysis methods suitable for forensic pharmaceutical analysis, mathematical calculations and statistical processing of the results.

The practical value of the results. The results obtained can be used to justify the choice of the method of determination of alprazolam in the forensic pharmaceutical analysis of materials cases.

Approbation of the research results. The materials of the qualification work were presented at the XXIX International Scientifical and Practical Conference of Young Scientists and Students TOPICAL ISSUES OF NEW MEDICINES DEVELOPMENT in the form of thesis:

The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis / R. Benothmane, I. V. Sych, O. V. Bevz, L. O. Perekhoda // Актуальні питання створення нових лікарських засобів:

матеріали XXIX міжнародної науково-практичної конференції молодих вчених та студентів (19-21 квітня 2023 р., м. Харків). – Kharkiv: NUPh, 2023. – P. 111-113.

The structure of the work. The work consists of an introduction, three chapters, general conclusions and list of references used, which is composed of 64 sources. Contents of work posted on 44 pages and contains 4 tables, 6 figures and 1 schema.

CHAPTER I
PHARMACOLOGICAL PROPERTIES OF CLONAZEPAM

1.1. Pharmacodynamic properties

Humans have been using hypnotic-sedative agents for any centuries. Barbiturates appeared in the 1930s, and the first benzodiazepine (chlordiazepoxide) was marketed in the early 1960s. Benzodiazepines are among the most commonly pre-scribed psychotropic medications worldwide and the prevalence of long-term use in the general population is 2% to 7%. The problem of use of benzodiazepines is related to the use of high doses and associated with the use of the drugs have been abuse, dependence, and withdrawal sequelae [1].

Since being first patented in 1960 and then released for sale from Roche in the US in 1975, clonazepam has experienced a storied history in the treatment of the aforementioned medical conditions. Now available as a generic medication, the agent continues to see exceptionally high use as millions of prescriptions are written for the medication internationally every year. Unfortunately, however, like most benzodiazepines, clonazepam use has also been associated with recreational use and drug abuse [2].

Clonazepam is indicated as monotherapy or as an adjunct in the treatment of Lennox-Gastaut syndrome (petit mal variant), akinetic, and myoclonic seizures. Furthermore, clonazepam may also be of some value in patients with absence spells (petit mal) who have failed to respond to succinimides. Additionally, clonazepam is also indicated for the treatment of panic disorder, with or without agoraphobia, as defined in the DSM-V.

Alternatively, some regional prescribing information note that clonazepam is indicated for all clinical forms of epileptic disease and seizures in adults, especially absence seizures (petit mal) including atypical absence; primary or secondarily generalised tonic-clonic (grand mal), tonic or clonic seizures; partial (focal) seizures with elementary or complex symptomatology; various forms of myoclonic seizures,

myoclonus and associated abnormal movements. Such regional label data also has clonazepam indicated for most types of epilepsy in infants and children, especially absences (petit mal), myoclonic seizures and tonic-clonic fits, whether due to primary generalized epilepsy or to secondary generalization of partial epilepsy

Clonazepam as observed with other benzodiazepines has hypnotic, sedative, anxiolytic, anticonvulsant, muscle relaxant, and amnesic properties. It is rapidly and completely absorbed after oral administration and extensively metabolized in the liver, primarily by cytochrome P450 (CYP) isoenzymes 3A4, to its major metabolite 7-aminoclonazepam which has weak anticonvulsant activity and is excreted mainly in urine. CYP3A4 is known to be involved in the metabolism of a wide variety of xenobiotics and has a large potential for drug interactions. This enzyme is also documented to exhibit a genetic polymorphism [3].

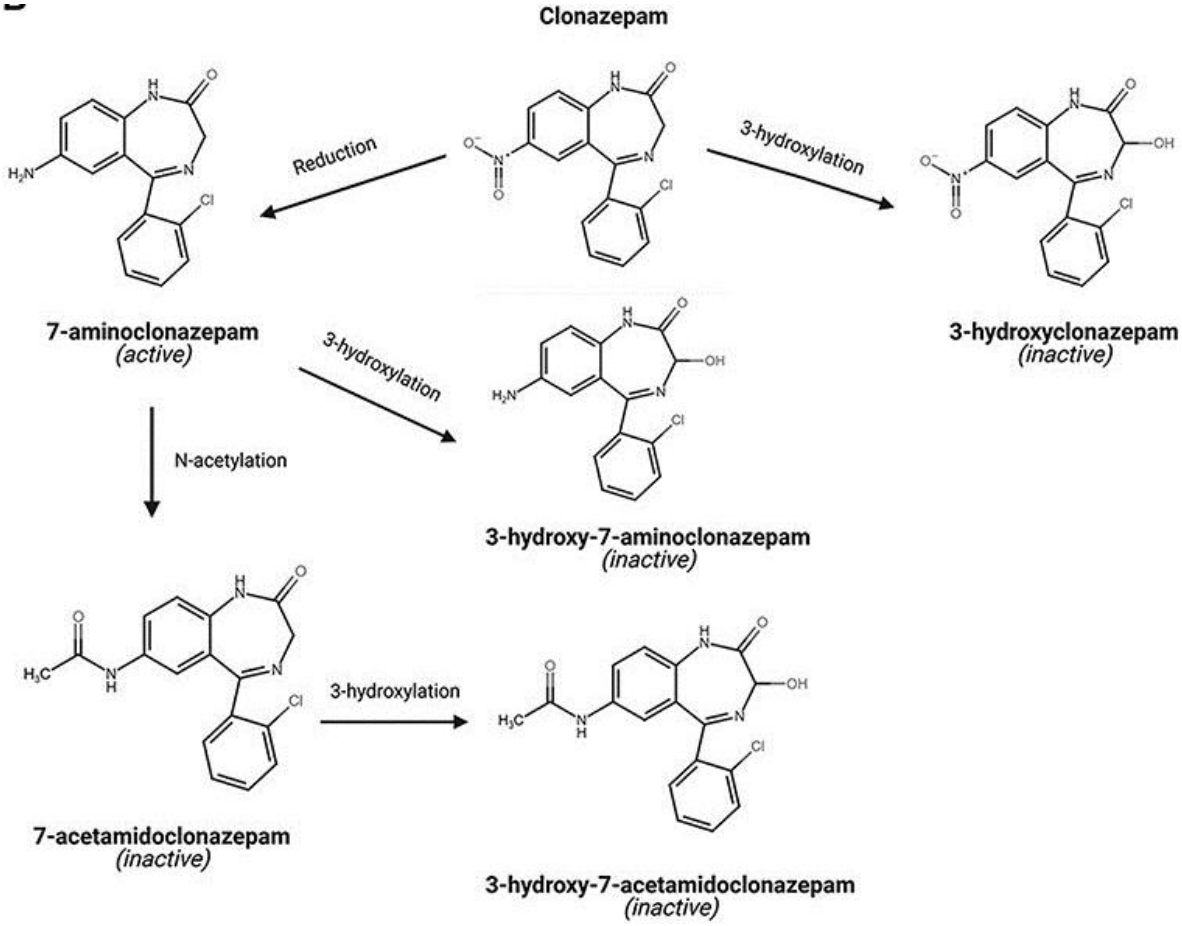
1.2. Pharmacokinetic properties

The core chemical structure of benzodiazepines consists of the combination of a benzene ring with a diazepine ring, with positions 1, 2, 5 or 7 available for substitution. Typically, the 5 position of the diazepine ring contains an aryl substituent. Additional substituents in the 1 and 4 positions of the diazepine ring lead to different 1,4-benzodiazepins [4].

Clonazepam is rapidly and completely absorbed after oral administration. The absolute bioavailability of clonazepam is about 90%. Maximum plasma concentrations of clonazepam are reached within 1 to 4 hours after oral administration. Clonazepam is approximately 85% bound to plasma proteins. Clonazepam is highly metabolized, with less than 2% unchanged clonazepam being excreted in the urine [5].

Clonazepam is extensively metabolized by nitro-reduction to 7-amino-clonazepam, which is further N-acetylated to 7-acetamido-clonazepam (Scheme 1.1.). Hydroxylation of these metabolites and the parent compound to the respective 3-hydroxy metabolites has also been reported; however, it is a minor metabolic

pathway. In human plasma, the hydroxylated metabolites were found to be present at very low concentrations or absent, whereas the concentration of 7-amino-clonazepam can be as high as or even higher than the concentration of clonazepam. Although no pharmacological activity is attributed to 7-amino-clonazepam, it has some affinity for benzodiazepine receptor and is able to compete with clonazepam. 7-Amino-clonazepam has been assumed to competitively modify the effect of clonazepam on GABA-A response and appears to exert mild effects as a partial agonist for the GABA-A receptor. Furthermore, high levels of 7-amino-clonazepam in patients have been associated with withdrawal symptoms. Nitro-reduction, the major route of clonazepam metabolism, is catalyzed by CYP3A enzymes, whereas N-acetyl transferase 2 (NAT2) is responsible for the acetylation of 7-amino-clonazepam.



Scheme 1.1. Metabolic pathways of clonazepam.

CYP3A4 activity displays more than 100-fold inter-individual variability, which is partly attributed to genetic factors. CYP3A4*1B allele leads to increased transcription; however, the clinical significance of CYP3A4*1B to CYP3A4 function is doubtful. CYP3A4*22 displays low hepatic CYP3A4 expression and results in decreased CYP3A4 activity. Elens et al. [6] suggested to evaluate the association between CYP3A4*22 and pharmacokinetic behavior of CYP3A substrates in combination with CYP3A5 genotype. CYP3A5*3 allele results in splicing defect and nonfunctional CYP3A5 protein. Those individuals who have functional CYP3A5 enzyme are presumed to metabolize some CYP3A substrates more rapidly than CYP3A5 nonexpressers. Human NAT2 is a polymorphic gene, generally dividing the population into slow and rapid acetylators [7]. NAT2*4 is the wild-type allele, responsible for rapid acetylator phenotype, whereas NAT2*5, NAT2*6, and NAT2*7, the most common alleles in white populations, are defined as slow acetylator alleles. The polymorphic CYP3A and NAT2 alleles may explain some inter-individual differences in blood concentrations of clonazepam and 7-amino-clonazepam; however, nongenetic factors (hormones, diseases, age, medication) can modify CYP3A4 activities, resulting in transient poor (or extensive) metabolism. The genotype determines the potential for the expression of functional or nonfunctional CYP enzyme, whereas nongenetic factors give rise to altered phenotypes. CYP3A4*1/*1 genotype, predicted to be translated to CYP3A4 enzyme with normal function, may be switched into poor (or extensive) metabolism due to phenoconversion [8]. Patients' clonazepam-metabolizing capacity can be estimated by the evaluation of CYP3A status and acetylator phenotype. Although CYP3A5 expressers and slow or rapid acetylators are simply identified by CYP3A5 and NAT2 genotyping, the crucial task is the assessment of hepatic CYP3A4 activity. We previously described a complex diagnostic system (CYPtest) that determines CYP3A-metabolizing capacity by the current CYP3A4 expression in leukocytes. CYP3A4 mRNA levels in leukocytes were proven to inform about the hepatic CYP3A4 activity. Patients with CYP3A4*22 allele are predicted to display decreased CYP3A4 mRNA levels, which lead to permanent low CYP3A4 activity,

whereas nongenetic factors modifying the expression of functional CYP3A4 gene result in transient poor (or extensive) clonazepam metabolism [9].

1.3. Toxicity

Potential problems associated with improper use or abuse of clonazepam include physical and psychological dependence, suicidal thoughts or actions, worsening of depression, sleep disorders, and aggression. Central nervous system depression and rarely cardiorespiratory depression characterize oral benzodiazepine overdoses. At clonazepam plasma concentrations >100 ng/mL additionally reflected in therapeutic doses, toxic symptoms such as drowsiness and ataxia can occur. Data on toxicological concentrations of drugs collected from the data indicate that 1000 ng/ml of clonazepam is a comatose-lethal concentration.

An increased risk of congenital malformations associated with the use of benzodiazepine drugs like clonazepam has been suggested in several studies. There may also be non-teratogenic risks associated with the use of benzodiazepines during pregnancy. There have been reports of neonatal flaccidity, respiratory and feeding difficulties, and hypothermia in children born to mothers who have been receiving benzodiazepines late in pregnancy. In addition, children born to mothers receiving benzodiazepines late in pregnancy may be at some risk of experiencing withdrawal symptoms during the postnatal period. In general, it is best for patients who are of childbearing potential and also use benzodiazepines like clonazepam to discuss such matters with their health care professionals as careful consideration must be undertaken regarding the intersection of the risks of untreated seizure potential in the patient and any possible toxicity to the fetus.

Although the active ingredient of clonazepam has been found to pass into the maternal milk in small amounts only, mothers receiving clonazepam should not breast-feed their infants.

Since the possibility that adverse effects on the physical or mental development of the child could become apparent only after several years, the risk-

benefit consideration of the long-term use of clonazepam in pediatric patients younger than five years of age is important.

The pharmacological effects of benzodiazepines like clonazepam appear to be greater in elderly patients than in younger patients even at similar plasma benzodiazepine concentrations, possibly because of age-related changes in drug-receptor interactions, post-receptor mechanisms, and organ function. In general, elderly patients should be started on the lowest possible dose of clonazepam and observed closely. There is an increased risk for falls and fractures among elderly and debilitated benzodiazepine users. The risk is increased in those taking concomitant sedatives, including substances like benzodiazepines, alcoholic beverages, and so on.

Some oral LD50 values documented are >4000 mg/kg for the mouse model, >4000 mg/kg for the adult rat model, and >2000 mg/kg for the rabbit model [10].

A case report of mega dose clonazepam dependence was reported by Mowla et al [11]. A 24-year-old woman was using 180 mg/day of clonazepam in 3 divided doses without any medical problem. The patient was able to tolerate such a high dose of clonazepam without evident impairment in psychomotor function, speech, orientation, and consciousness. She only experience dun controllable tension and insomnia after tapering down clonazepam. However, serum concentrations of clonazepam were not stated. Because tolerance to clonazepam develops in many patients, it has been difficult to identify a clear correlation between serum levels of clonazepam and either efficacy or toxicity. In patients with epilepsy treated with therapeutic doses of clonazepam, serum concentrations in the order of 20 to 70 ng/mL have been reported and drug concentrations above the recommended reference range that causes the laboratory to feedback immediately to the prescribing physician (i.e., laboratory alert level) is 80 ng/mL.15,16 The therapeutic reference range / recommended drug concentration of clonazepam used as anxiolytic/hypnotic drugs is 4 to 80 ng/mL (the ‘‘laboratory alert level’’ is 100 ng/mL) [12, 13].

In addition to the therapeutic use, they can produce sedative and amnestics effects: an intravenous dose of short acting benzodiazepines assists in the induction

of surgical anesthesia. In a recent review on Drug-facilitated sexual assault, with a total of 22 studies included, covering toxicological findings in Drug-facilitated sexual assault cases in North America, Europe, Asia, South Africa and Australasia, a variety of benzodiazepines were observed, with the most common being diazepam, clonazepam, alprazolam, and oxazepam [14, 15] The majority of cases involved women (87% -100%). Specifically, hypnotics were found in 10 studies primarily as zopiclone or zolpidem. Benzodiazepines were detected in all studies with a range from 3.5% to 82% of total cases. In Europe, benzodiazepines were detected in 5.1% to 82.0% of cases and the most frequently detected benzodiazepines and hypnotics were diazepam, alprazolam, oxazepam, clonazepam, temazepam, and zolpidem [16]. In Asia, it was nimetazepam, flunitrazepam, clonazepam, lorazepam, and the designer benzodiazepines diclazepam and flualprazolam. In the United States, it was oxazepam, diazepam, clonazepam, alprazolam and lorazepam. Lastly, lorazepam was the most common benzodiazepine detected in Canada, while diazepam was the most common found in Australia and New Zealand. (Figure 1.1.).

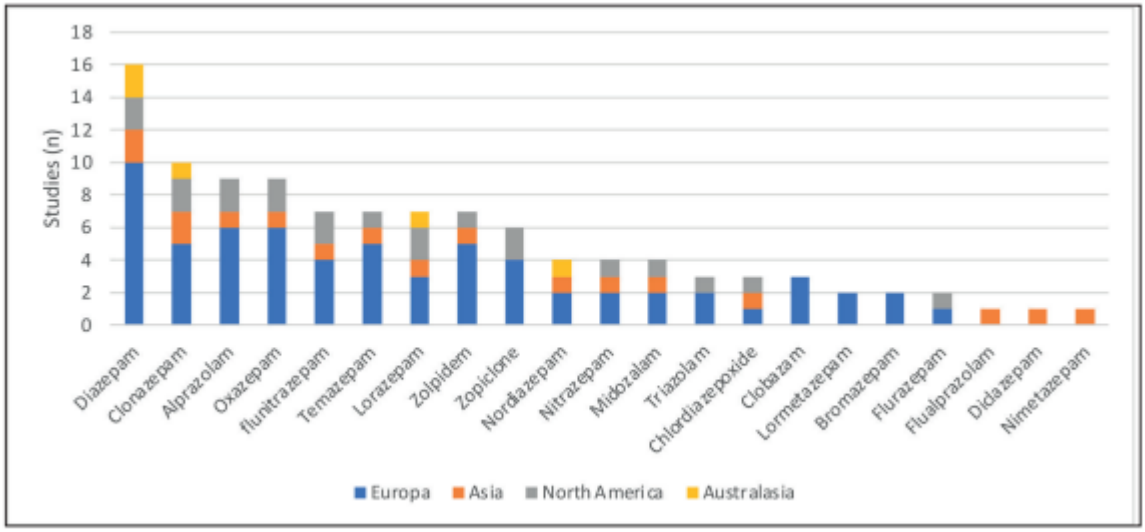


Figure 1.1. Benzodiazepines, designer benzodiazepines and hypnotics detected in toxicological analysis of drug-facilitated sexual assault cases.

In Europe, the most frequently detected benzodiazepines and hypnotics were diazepam, alprazolam, oxazepam, clonazepam, temazepam, and zolpidem (Figure 1.1). In Asia, it was nimetazepam, flunitrazepam, clonazepam, lorazepam, and the

designer benzodiazepines diclazepam and flualprazolam. In the United States, it was oxazepam, diazepam, clonazepam, alprazolam and lorazepam. Lastly, lorazepam was the most common benzodiazepine detected in Canada, while diazepam was the most common found in Australia and New Zealand [17, 18].

Diazepam, clonazepam, alprazolam and oxazepam were the most commonly detected benzodiazepines across studies, though this could be due to their long half-life. Consequently, it is difficult to estimate whether these are indeed the most common benzodiazepines used in drug-facilitated sexual assault. While most studies reported drugs of abuse and benzodiazepines to be the most prevalent in toxicological findings, the limited contextual information makes it complicated to interpret the toxicological findings and estimate level of covert drug administration.

Many crimes, especially of a sexual nature, are committed using sedative substances to reduce the victim's state of consciousness and reactivity and are defined as "drugs facilitated crimes". Among these, benzodiazepines and some new designer derivatives are widely used especially in liquid formulations added to other drinks.

Detection of Benzodiazepines is crucial in the investigation of drug-facilitated sexual assault. The presence of Benzodiazepines in a sample from a victim who does not take prescribed medication to treat anxiety, insomnia, panic disorder or seizures should be considered a relevant finding. Additionally, the blood concentration of Benzodiazepines is also important as higher doses are generally correlated with a higher degree of impairment·[19].

One of the major analytical challenges associated with the analysis of Benzodiazepines in body fluids is the low concentrations of the parent drug. The highly potent flunitrazepam has a therapeutic dose of 0.5–2 mg, which translates to a blood concentration of ca. 0.005–0.015 ng/mL for the therapeutic range and a lower limit of 0.05 ng/mL for the toxic range [20]. Furthermore, some Benzodiazepines exhibit a short elimination half-life as illustrated with the half-life of midazolam, i.e., ca. 1.5–3 h. Finally, the concentrations are sometimes below the limits of detection by the time the biological samples are collected, especially when

a long time has elapsed between the alleged assault and the moment that the victim contacts the authorities.

The number of Benzodiazepines and Benzodiazepines that can be used in a sexual assault represents another challenge. Indeed, a large number of Benzodiazepines with a different structure, metabolism and excretion are available in the (black) market, requiring very selective analytical methods capable of discriminating and identifying these compounds. Moreover, some of the Benzodiazepines are positional isomers (e.g., diclazepam and 4-chlorodiazepam), and their separation requires highly selective methods.

Benzodiazepines pose additional challenges in the analysis and interpretation of the data. First, reference standards are often not commercially available. Moreover, the limited amount of clinical data available on the effects of Benzodiazepines makes it difficult to establish cut-off values for the detection of a one-time drug intake for the purpose of chemical submission. Finally, little is known on the metabolism of Benzodiazepines and the associated metabolites, which renders the development of comprehensive analytical approaches very challenging, especially for the analysis of urine samples.

CONCLUSION

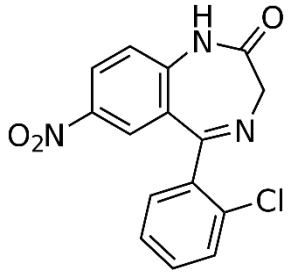
1. The pharmacokinetic and pharmacodynamic properties of clonazepam are reviewed.

2. Clonazepam is one of the most commonly detected benzodiazepines across toxicology and forensic studies, though this could be due to their long half-life. The mechanism of action and metabolism of the drug, which can lead to toxicity, are therefore considered.

CHAPTER II
PHARMACEUTICAL INFORMATION: PHYSICOCHEMICAL PROPERTIES AND METHODS OF DETERMINATION OF CLONAZEPAM

2.1. Structural formula and physiochemical properties

Structural formula:



Proper name:

Clonazepam

Chemical name:

5-(2-chlorophenyl)-1, 3-dihydro-7-nitro-2H-1, 4-benzodiazepin-2-one.

Molecular formula:

$C_{15}H_{10}ClN_3O_3$

Molecular mass:

315.7

Appearance:

slightly yellowish, crystalline powder

Solubility:

sparingly soluble in acetic anhydride and in acetone, slightly soluble in methanol and in ethanol (95), very slightly soluble in diethyl ether, and practically insoluble in water

Melting point:

about 239 °C

2.2. Methods of pharmaceutical analysis of clonazepam

According to the monograph of Europe Pharmacopoeia, the identification substance proposes by method spectroscopy in infrared region. By comparing the obtained spectrum with the spectrum of a standard sample or the spectrum of an electronic database (Figure 2.1.) [21].

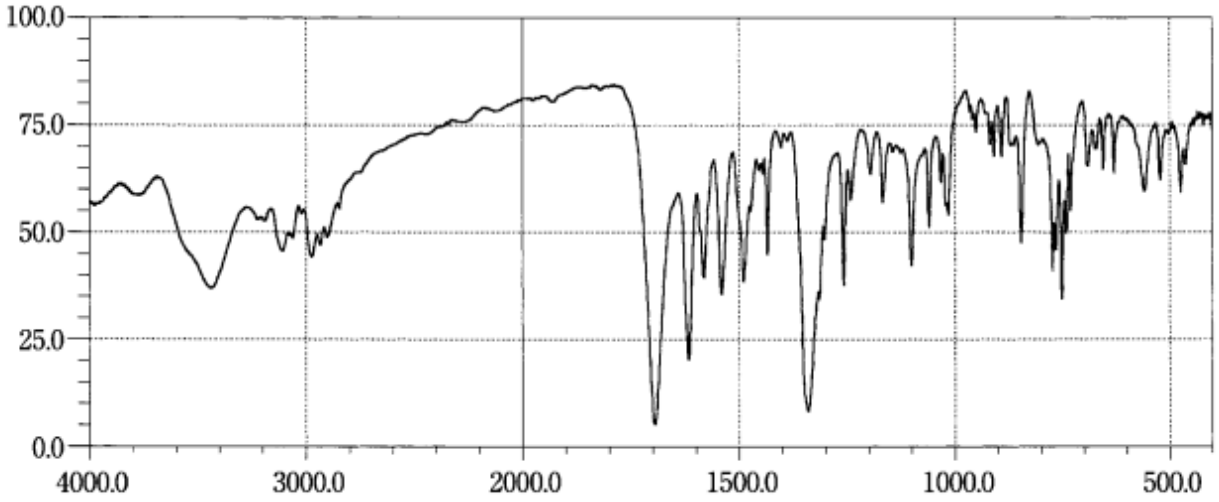


Figure 2.1. Infrared reference spectra of clonazepam [22]

According to Japanese Pharmacopoeia, one of the methods of identification of clonazepam is absorption spectroscopy in ultraviolet region. Determine the absorption spectrum of a solution of Clonazepam in methanol (1 in 100,000) and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths: it exhibits a maximum between 307 nm and 311 nm. (Figure 2.2.) [23].

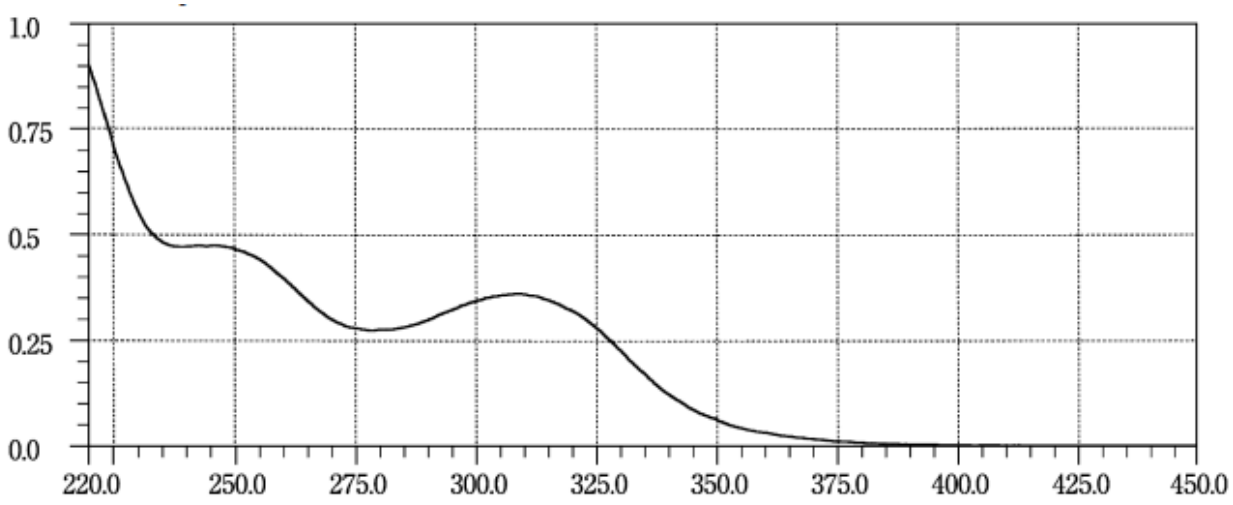


Figure 2.2. Ultraviolet reference spectra of methanol solution of clonazepam [24].

According to the methods offered by Sigma Aldrich, the determination of clonazepam is also recommended by the method of UV spectroscopy, but a 0.2 N solution of sulfuric acid is used as a dissolution medium (Dilution: 1:199 (v/v). Under these conditions, the maximum light absorption of the tested solution observed at a wavelength of 275 nm (Figure 2.3.).

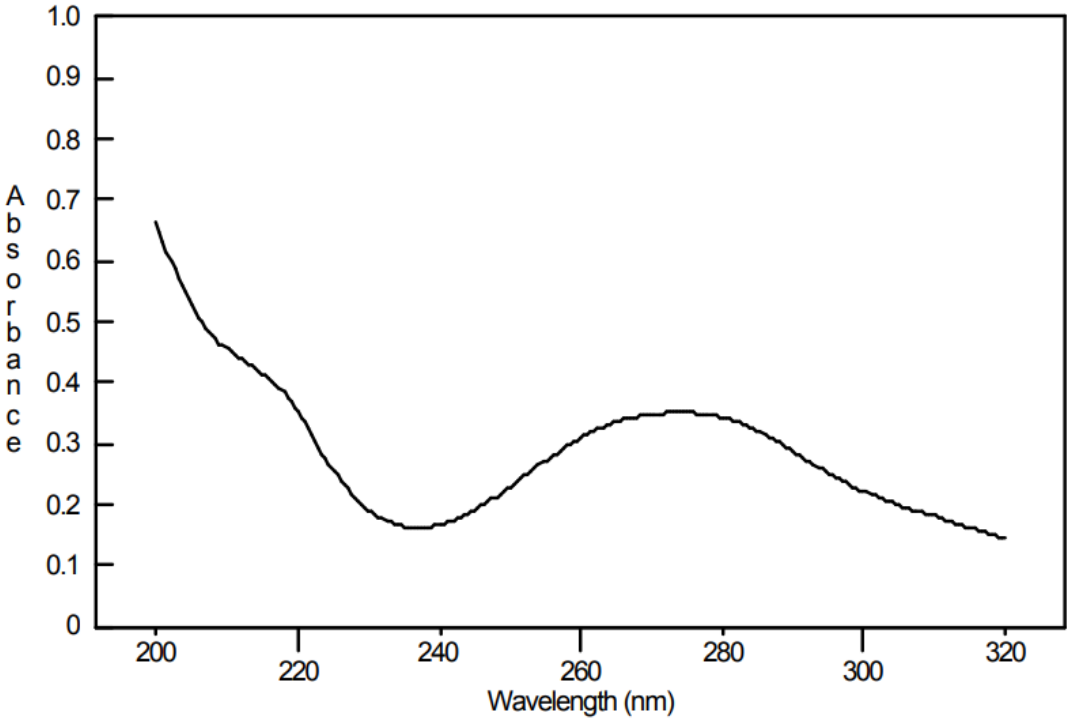


Figure 2.3. Ultraviolet reference spectra of methanol solution of clonazepam in 0,2 N solution of sulfuric acid [25]

For the quantitative determination of clonazepam in a substance, the European Pharmacopoeia recommends the acid-base titration method in non-aqueous solvents. The end point of the titration is determined by potentiometric method.

2.3. Methods of determining clonazepam in toxicological analysis

Clonazepam has a plasma half-life varying from 19 to 60 h, where the mean value is 40 h. It is extensively metabolized in the liver, primarily by CYP3A4, to its major metabolite 7-aminoclonazepam. 7-aminoclonazepam is excreted mainly in urine. Only about 0.5% of parent drug is excreted unchanged in the urine. In clinical studies oral administration of a single 2 mg dose resulted in an average plasma concentration of 17 ng/mL (range: 7-24 ng/mL) of Clonazepam between 1 and 4 h after ingestion. In studies of patients receiving 6 mg/day chronic therapy, the plasma concentration of Clonazepam and 7-aminoclonazepam were reported as 29-75 ng/mL and 23- 137 ng/mL, respectively [26]. Because of above metabolic characteristics, sometimes parent drug Clonazepam cannot be directly detected while only its major metabolite 7-aminoclonazepam can be detected. This indicated the detection of 7-aminoclonazepam can be regarded as evidence of Clonazepam intake. It has been reported that postmortem bioconversion to 7-aminometabolites may also occur, to the extent that little or no parent drug may be present; the identification and quantification of the 7-amino metabolites are of importance toxicologically as they are often the only indication of nitrobenzodiazepine use prior to death. Consequently, the simultaneous determination of Clonazepam and its metabolite 7-aminoclonazepam is very important in the fields of forensic toxicology [27].

On the other hand, in most cases, victims don’t often report until after some time; therefore, blood and urine samples are often detected more than 24-72 h after ingestion. Moreover, only immunoassay screening and sometimes liquid chromatography diode array detector and gas chromatography-mass spectrometry analysis are performed on blood and urine at the hospital laboratory where the victim

is admitted. Since most of the drugs involved in DFC are not detectable at low levels by these techniques, resulting false negative results may lead to an inaccurate conclusion and to premature destruction of samples.

For this reason, it is important to develop highly sensitive and specific analytical methods for the simultaneous determination of Clonazepam and its metabolite 7-aminoclonazepam in biological fluids. Numerous analytical methods including spectrophotometry, gas chromatography, gas chromatography-mass spectrometry and high-performance liquid chromatography have been presented for testing Clonazepam in biological fluids. However, when these methods are used, some problems of low sensitivity, specifi city and reproducibility are encountered [28]. Due to its ability to analyze thermally unstable or polar compounds, such as 7-aminoclonazepam, liquid chromatography-based methods offer a distinct advantage over gas chromatography related techniques. Recently, detection of Clonazepam and its metabolite 7-aminoclonazepam in biological fluids has become available with the development of more and more sensitive LC-MS apparatuses. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as the most suitable tool to test for low concentrations of Clonazepam and its metabolite 7-aminoclonazepam. At present, LC-MS/MS is widely used in the determination of benzodiazepines and their related drugs due to its strong specificity, high sensitivity, good reproducibility, wide linear dynamic range and other characteristics [29]. But LC-MS/MS can only analyze the target compound, which is included in established database, while it can't screen the unknown compound which is not included in established database no matter how much concentration of the unknown compound is. These lacks LC-MS/MS assays limit qualitative analysis and rapid screening of target compound containing multiple components at trace levels (e.g., ng/L) [30]. Recent advances in LC/MS technology have led to the availability of time-of-flight (TOF) LC/MS systems. These provide a greater level of the analyte information as a result of high resolution and the ability to collect accurate mass information to the sub ppm levels [31]. This significantly increases the confidence in the analyte identification by limiting the possible number of candidate compounds. The

coupling of a quadrupole and a collision cell to the TOF analyzer, the fragmentation of pre-selected ions and the identifi cation of compounds based on their product ion spectra. Accurate mass determination of both the precursor and product ions is therefore possible [32, 33]. Rapid screening and confirmation of the analyte can be achieved with MS matching scores, deviation of retention time, measured mass, isotopic abundance matching scores and isotope spacing match scores and MS/MS matching scores. Except for some immunoassays, liquid liquid extraction and solid phase extraction [34] are commonly included in sample pretreatment methods for the determination of Clonazepam and 7-aminoclonazepam in biological fluids. Compared with liquid liquid extraction and solid phase extraction can improve the extraction recovery of Clonazepam and 7-aminoclonazepam in biological fluids due to its advantages such as less use of organic solvents, stronger selectivity, shorter separation time, better reproducibility and better the clean-up effect. Furthermore, a large-diameter extraction disk is equipped with fully automatic solid phase extraction system for solid phase extraction, and the sample pretreatment procedure becomes simple and efficient.

Analytical methods

Immunoassays

Several immunoassays can be considered for the detection of Benzodiazepines, including the enzyme multiplied immunoassay technique, enzyme-linked immunosorbent assay, homogenous enzyme immunoassay, kinetic microparticle immunoassay, and cloned-enzyme donor immunoassay. Due to their speed and ease of use, these immunoassays are typically used as a screening method prior to confirmation using gas chromatography or liquid chromatography combined with MS.

Immunoassays are broadly used in forensic toxicology due to their ease of use, rapidity, flexibility, and the possibility to obtain semi-quantitative results. Many of these assays are flexible enough to react to different Benzodiazepines structures. However, in some cases, the large diversity of chemical structures of Benzodiazepines leads to false-positive or false-negative results due to the variable

immunoreactivity of the antibodies used for the immunoassays. As an example, the EMIT® II Plus benzodiazepine assay [35] uses polyclonal sheep antibodies targeting diazepam as binding site. The drug present in the biological sample compete with a labeled diazepam that is added as substrate in the assay. Moreover, even if assays are able to distinguish between positive and negative specimens, the semi-quantitative information is approximate, as it reflects the cumulative concentrations of the drugs and their metabolites, which cross-react with the assay [36].

Since the structures of Benzodiazepines are similar to those of Benzodiazepines, immunoassays may also enable their detection in biological samples. Pettersson Bergstrand et al. evaluated four different assays, cloned-enzyme donor immunoassay, enzyme multiplied immunoassay technique II plus and homogenous enzyme immunoassay for the detection of 13 Benzodiazepines in urine samples [37]. Generally, a high cross-reactivity was shown for Benzodiazepines in all assays, demonstrating that conventional kits can be used for the standard urine immunoassay screening of Benzodiazepines. However, flutazolam, meclonazepam and nifoxipam showed a low cross-reactivity in the enzyme multiplied immunoassay technique II plus assay, similar to deschloroetizolam, etizolam and flutazolam when using the homogenous enzyme immunoassay.

It is worth mentioning that Benzodiazepines with a more divergent structure compared to conventional Benzodiazepines might remain undetected during the screening phase. Moreover, these assays have been evaluated using parent drugs; some metabolites may not cross-react using these antibody-based tests. Finally, even in the presence of a sufficient cross-reactivity, the low concentrations expected from potent Benzodiazepines in blood after an alleged DFSA incident might not be detected by these methods—similar to Benzodiazepines.

MS-based techniques

Gas chromatography –MS and Liquid chromatography –MS are typically used for confirmation after the initial immunoassay-based screening, as those techniques provide the high sensitivity, selectivity and accuracy needed for the quantitation of drugs and associated metabolites in biological samples. Nowadays,

many routine laboratories also use MS-based approaches as initial screening techniques. Table 2.1 lists the MS-based analytical methods developed in the last decade for the analysis of Benzodiazepines in forensic samples, which are blood, urine and hair. The large majority of applications report the use of liquid chromatography –MS, which does not require the tedious and time-consuming sample preparation required prior to gas chromatography –MS analysis.

Table 2.1.

Analytical methods applied to the analysis of benzodiazepines in biological matrices from prospective forensic and toxicological samples

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Analytical method	Limits of detection	Reference
18 BZDs (1 metabolite) and other drugs Total: 46 compounds	Whole blood	Adjust to pH 4 (ammonium acetate buffer)	SPE Sorbent: Oasis® MCX Elution: ethyl acetate (5% ammonium hydroxide)	UHPLC–TOF-MS	LOQ: 0.1–3 ng/g	[37]
24 BZDs (7 metabolites) and other drugs Total: 65 compounds	Whole blood	Deproteinization with methanol Adjust to pH 9 (saturated carbonate buffer)	DLLME Extractant: Chloroform Disperser: Methanol with addition of sodium chloride	UHPLC–MS-MS Acquisition: SRM	LOD: 2 ng/mL LOQ: 5 ng/mL except for alprazolam and chlordiazepoxide	[38]
23 BZDs (2 metabolites) and other drugs Total: 96 compounds	Whole blood		PP and PLR Protein precipitation with acetonitrile/methanol (95:5, v/v), supernatant: formic acid 1% in acetonitrile (v/v) on Phree® Phospholipid Removal Plates	HPLC–MS-MS Acquisition: SRM	LOD: 0.1–10 ng/mL	[39]

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Analytical method	Limits of detection	Reference
18 BZDs (1 metabolite) and other drugs Total: 128 compounds	Urine	Hydrolysis with β-glucuronidases/arylsulfatase at pH 5.5 (acetate buffer) at 55℃ for 1 h	SPE Sorbent: Oasis® HLB Elution: methanol and methanol/isopropanol (3:1, v/v)	GC--EI-MS Derivatization: BSTFA + 1% TCMS in ethyl acetate/acetonitrile (1:1, v/v)	LOD: 120–12,000 ng/mL LOQ: 200-20,000 ng/mL	[40]
21 BZDs (4 metabolites) and Z-drugs Total: 23 compounds	Urine	Hydrolysis with β-glucuronidase at pH 6.0 at 55℃ for 1 h, then alkalization at pH 7.5 with phosphate buffer	LLE Dichloromethane/propan-2-ol mixture (85:15, v/v)	HPLC–MS-MS Acquisition: SRM	LOD: 0.5–30 ng/mL LOQ: 2–100 ng/mL	[41]
5 BZDs (2 metabolites)	Urine	Adjust to pH 9.5 (carbonate buffer)	LLE Ethyl acetate	HPLC–MS-MS Acquisition: MRM	LOD: 0.125–1 ng/mL LOQ: 0.25–5 ng/mL	[42]
22 BZDS (4 metabolites) and other drugs	Urine	Both hydrolyzed and nonhydrolyzed samples Hydrolysis with β-glucuronidase at 55℃ for 30 min	Directly (without extraction)	HPLC–MS-MS Acquisition: MRM	N/S	[43]

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Analytical method	Limits of detection	Reference
Total: 91 compounds						
24 BZDs (5 metabolites) and other drugs Total: 54 compounds	Urine	Adjust to pH 9.5 (sodium bicarbonate buffer)	LLE Ethyl acetate	HPLC–MS-MS Acquisition: MRM	LOD: 0.5–5 ng/mL LOQ: 1–10 ng/mL	[44]
18 BZDs (6 metabolites)	Hair	Segmentation and rinsing twice with dichloromethane. Drying and segments cut into pieces <3 mm and pulverized in phosphate buffer (pH 8.4 at RT for 1 h)	LLE Dichloromethane	HPLC–MS-MS Acquisition: MRM	LOD: 0.0005–0.002 ng/mg	[45]
18 BZDs (2 metabolites) and other drugs Total: 35 compounds	Hair	Decontamination with two washes with water followed by two washes with dichloromethane. Drying and segmentation into	LLE Dichloromethane/ether (70:30, v/v).	HPLC–MS-MS Acquisition: MRM	LOD: 0.0005–0.01 ng/mg LOQ: 0.0005–0.01 ng/mg	[46]

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Analytical method	Limits of detection	Reference
		<1 mm. Incubation overnight at RT with phosphate buffer pH 5.				
13 BZDs (3 metabolites) and other drugs Total: 52 compounds	Hair	10 mg hair, one wash with isopropanol and two with H_2O. Drying and extraction. Segmentation in 1–2 mm segments or pulverization. Incubation overnight at 37°C	Extraction Methanol:acetonitrile:ammonium formate (pH 5.3) Filtration PTFE filter	UHPLC–TOF-MS	LOD: 0.01–0.04 ng/mg LOQ: 0.05 ng/mg	[47]
20 BZDs (6 metabolites) and 3 Z-drugs Total: 23 compounds	Hair	Decontamination with H_2O, acetone and hexane. Pulverization in a bench-top mill.	Extraction under shaking 1st step: methanol 2nd step: methanol/ammonium formate buffer (1:1, v/v) at pH 3.5	UHPLC–MS-MS Acquisition: MRM	LOQ: 0.0005–0.01 ng/mg	[48]
28 BZDs (9 metabolites) and other drugs	Blood	Deproteinization with methanol, adjust to pH 9 (saturated carbonate buffer)	DLLME Extractant: chloroform Disperser: methanol with addition of sodium chloride	UHPLC–MS-MS Acquisition: MRM	LOD: 2 ng/mL LOQ: 5 ng/mL except for	[49]

Target analytes	Biological matrix	Sample pretreatment	Analyte extraction	Analytical method	Limits of detection	Reference
Total: 200 compounds					alprazolam and chlordiazepoxide	
	Urine	Hydrolysis with β-glucuronidase in acidic medium	Dilution and shoot Aqueous formic acid	UHPLC–MS-MS Acquisition: MRM	LOD: 1–5 ng/mL (not determined for hydroxymidazolam)	
	Hair	Decontamination with two washes with aqueous tween 80 and one wash with acetone. Segmentation into 1 mm.	Extraction Aqueous formic acid at 40°C overnight	UHPLC–MS-MS Acquisition: MRM	N/S	

DLLME: dispersive liquid–liquid microextraction; LLE: liquid–liquid extraction; LOD: limit of detection; LOQ: limit of quantitation; PP: protein precipitation; RT: room temperature

Biosamples and sample preparation

Conventional matrices used for the detection of Benzodiazepines and Benzodiazepines include blood and urine (Table 2.1). Since many Benzodiazepines and their respective Phase I metabolites are conjugated with glucuronic acid during their Phase II metabolism, a deconjugation step is often performed prior to the sample preparation to enhance the detection of the nonconjugated analyte and improve the overall sensitivity. This deconjugation step typically consists of enzymatic hydrolysis using β-glucuronidase or a mixture of β-glucuronidase and arylsulfatase. Alternatively, the conjugated metabolites can also be analyzed, rendering the deconjugation step unnecessary [50].

Hair is an interesting alternative matrix for the analysis of Benzodiazepines as it allows for a longer detection window. Compared with blood and urine matrices, hair requires an extensive pretreatment procedure before the extraction of the target analytes. A decontamination step of hair fibers is required, prior or after segmentation or pulverization of the hair. Hair pulverization usually leads to higher extraction recoveries [51, 52]. The pulverized or segmented hair is then incubated with different solvent mixtures (up to 24 h) prior to the extraction step [53].

The sample preparation step relies on common procedures, including protein precipitation, liquid–liquid extraction (LLE), solid-phase extraction (SPE) and—for urine samples—dilute and shoot approaches. With the basic pKa of Benzodiazepines being in the range of approximately 1.5–3.5, alkalization of the samples prior to LLE is typically performed, prior to extraction using an adequate solvent, often ethyl acetate or dichloromethane. Rossi et al. used a miniaturized approach, that is, dispersive liquid–liquid microextraction for the extraction of Benzodiazepines and other relevant drugs. Dispersive liquid–liquid microextraction uses a combination of an extracting solvent (e.g., dichloromethane) and a dispersion solvent miscible with water (e.g., methanol), which is added to the sample. This ternary system leads to the formation of microdroplets, which strongly enhance the surface area and allows for instantaneous equilibrium partitioning of analytes between the aqueous sample

and the extraction solvent, resulting in a drastically reduced extraction time (i.e., a couple of seconds) [54].

Another interesting miniaturized approach is offered with parallel artificial liquid membrane extraction (PALME), used by Vårdal et al. for the extraction of Benzodiazepines, Benzodiazepines and Z-drugs in whole blood. In PALME, targeted analytes present in an aqueous donor solution are transferred through an organic supported liquid membrane to an acceptor solution. By modifying the pH of the donor and acceptor solutions, target analytes can be extracted and preconcentrated in a miniaturized and semi-automated format. By combining this setup with ultra-high pressure liquid chromatography--MS-MS analysis, limits of detection down to 0.1 ng/mL were achieved [55].

Separation and detection

As illustrated in Table 2.1, a large majority of Benzodiazepines analysis methods is based on LC–MS. Only one study reports the use of GC–MS for the analysis of Benzodiazepines (and other date-rape drugs) in urine samples. Most of the studies included the quantitation of Benzodiazepines and, therefore, frequently include tandem MS (MS-MS) approaches. Very few articles report the use of high-resolution MS, for instance, time-of-flight or Orbitrap mass analyzers.

The developed methods typically aim for the detection of concentrations lower than the therapeutic level, which seems to be easily achievable with state-of-the-art triple quadrupole instruments and adequate sample preparation.

It is worth mentioning that the isotopically labeled internal standards used for the accurate quantitation of Benzodiazepines were most frequently not analyte-specific, which may be explained by the low number of commercially available labeled Benzodiazepines standards [56].

CONCLUSION

1. The physicochemical properties of clonazepam are considered.
2. The literature on modern methods of clonazepam analysis, in particular for the identification, determination of purity and quantification of clonazepam in the substance and finished medicinal products, was studied.
3. Among the modern methods of pharmaceutical analysis, preference is given to physicochemical methods for the determination of clonazepam in pharmaceutical and forensic analysis.

CHAPTER III
CHOICE OF METHODS FOR THE DETERMINATION OF CLONAZEPAM BY PARAMETERS: ENVIRONMENTAL FRIENDLINESS, EFFICIENCY AND SELECTIVITY

Clonazepam is a benzodiazepine derivative that was approved for use as an anticonvulsant in the US in 1975. At present, Clonazepam has been one of the most frequently prescribed psychoactive drugs worldwide due to its hypnotic, anxiolytic, anticonvulsant and muscle-relaxant properties. However, Clonazepam, because of its pharmacological effects has been identified as a compound frequently used in drug-facilitated crimes such as robberies and sexual assaults in recent years [57].

The selection of a sensitive, specific and rapid method for the determination of clonazepam in forensic and pharmaceutical cases is therefore relevant.

The aim of this work is to choose a highly sensitive and specific analytical method for qualitative and quantitative analysis of Clonazepam as forensic and pharmaceutical object considering the cost, specificity and environmental friendliness of the proposed methods.

This study details the use of pharmacopeial method - Liquid chromatography in comparison to ultra-performance liquid chromatography–tandem mass spectrometry for determination Clonazepam in pharmaceutical and forensic materials.

3.1. Liquid chromatography method

For the determination of clonazepam in pharmaceutical and forensic materials, we consider the method of liquid chromatography, according to the monograph of the United States Pharmacopoeia for substances and finished drugs with clonazepam.

Buffer solution - Transfer about 6.6 g of anhydrous dibasic ammonium phosphate to a 1-L volumetric flask, dissolve in 950 mL of water, adjust with 1 N

phosphoric acid or 1 N sodium hydroxide to a pH of 8.0, dilute with water to volume, and mix.

Mobile phase - Prepare a filtered and degassed mixture of *Buffer solution*, methanol, and tetrahydrofuran (60:52:13). Make adjustments if necessary.

Diluent - Prepare a mixture of water, methanol, and tetrahydrofuran (60:52:13).

Standard preparation - Dissolve an accurately weighed quantity of USP Clonazepam RS in *Diluent*, and dilute quantitatively, and stepwise if necessary, with *Diluent* to obtain a solution having a known concentration of about 0.1 mg per mL.

System suitability solution - Dissolve suitable quantities of USP Clonazepam RS in *Diluent* to obtain a solution containing about 0.04 mg per ml.

Assay preparation - Transfer about 10 mg of Clonazepam, accurately weighed, to a 100-mL volumetric flask, dissolve in and dilute with *Diluent* to volume, and mix.

Chromatographic system - The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 15-cm column that contains packing L7. The flow rate is about 1 mL per minute. Chromatograph the *System suitability solution*, and record the peak responses as directed for *Procedure:* the relative retention times are about 2.2 for clonazepam related compound A, 2.5 for clonazepam related compound B, and 1.0 for clonazepam; and the resolution, *R*, between clonazepam related compound A and clonazepam related compound B is not less than 2.0. Chromatograph the *Standard preparation*, and record the peak responses as directed for *Procedure:* the tailing factor is not more than 1.5, and the relative standard deviation for replicate injections is not more than 2.0%.

Procedure - Separately inject equal volumes (about 50 µL) of the *Standard preparation* and the *Assay preparation* into the chromatograph, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity, in mg, of $C_{15}H_{10}ClN_3O_3$ in the portion of Clonazepam taken by the formula:

$$100C(r_U/r_S)$$

in which C is the concentration, in mg per mL, of USP Clonazepam RS in the *Standard preparation;* and r_U and r_S are the peak responses obtained from the *Assay preparation* and the *Standard preparation*, respectively [58].

This method has also been described in the works of scientists for the determination of clonazepam in biological fluids and medicines, so it is promising for consideration for use in pharmaceutical and forensic analysis [59].

3.2. Ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) method

The large majority of studies report the use of ultra-performance liquid chromatography – MS (Table 2.1) for the determination of clonazepam and even its metabolites in forensic and toxicological materials, which does not require the tedious and time-consuming sample preparation required before gas chromatography –MS analysis, for example, which is more often used in forensic analysis.

The clonazepam solution is prepared or purchased as methanolic solutions (1 mg/mL) from LGC Standards (Teddington, UK). Mixed working solutions for the clonazepam are prepared by subsequent dilution with water.

The solvents which use for mobile phases are LC–MS grade and purchased from Sigma–Aldrich (Poole, UK).

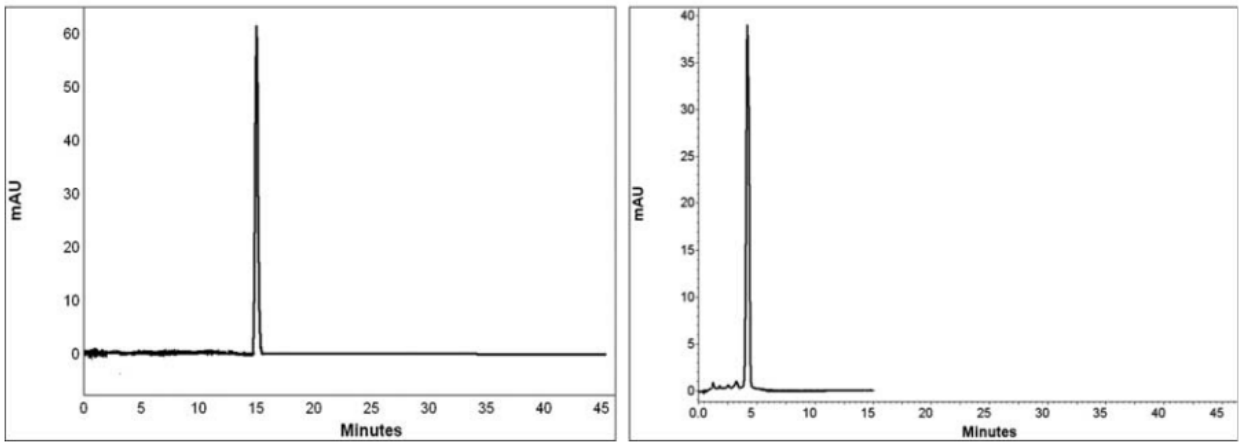
Deutera A mixed working internal standard (IS) solution is prepared daily by dilution of the deuterated analogues into mobile phase A at a concentration of 5 μg/mL. The controls, calibrators or specimens (0.30 mL) are spiked with 10 μL IS solution before the addition of 150 μL borate buffer (prepared from a saturated solution of disodium tetraborate decahydrate). The samples are vortex-mixed and then extracted with 0.90 mL of dichloromethane–ether–hexane (30:50:20 containing 0.5% isoamyl alcohol), and centrifuged at 3,000 rpm for 5 min. The supernatant is then transferred to a clean Eppendorf tube and dried on a Techne Driblock (Bibby

Scientific Limited, Stone, UK) at 40°C under nitrogen gas. The samples are reconstituted in 50 µL 80% water and 20% methanol, (6×concentration step) for injection onto the UPLC–MS-MS.

The samples are analyzed using a Waters Acquity UPLC and TQ detector using positive electrospray ionization and multiple reaction monitoring mode. The capillary voltage is 3 kV and the desolvation temperature is 400°C. The desolvation gas flow is set at 800 L/h and the source temperature is 120°C. An Acquity UPLC BEH C18 column with dimensions of 2.1 × 100 mm and a 1.7-µm particle size is used; column temperature is maintained at 50°C. The mobile phase flow rate was 0.40 mL/min, and the injection volume is 10 µL. The mobile phase is 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). A gradient separation is used starting at 70% A–30% B, ramping to 65% B over 2.5 min, 70% B over 0.75 min, 77% B over 0.75 min, then to 95% B over 0.05 min and holding at 95% B for 1 min, returning to 30% B and re-equilibrating for 2 min before the next injection (4.5 min analysis time, 7.5 min injection to injection). The precursor ions, product ions, cone voltages and collision energies for the analyte and IS are listed in Table 3.1. Extracted ion chromatograms (quantifier trace for an extracted 1 ng/mL serum calibrator) for all analytes over the 4.5-min analysis time are shown in Figure 3.1 (b).

3.3. Comparison of the analysis results of the proposed methods

Comparison of chromatograms of clonazepam in HPLC and UPLC–MS-MS is shown in Figures 3.1.



a b

Figure 3.1. Comparison of chromatograms of clonazepam in HPLC (a) and UPLC–MS-MS (b)

Table 3.1.

Mass spectrometry parameters for clonazepam

Analyte	Precursor (m/z)	Cone voltage (V)	Product 1 (m/z)	Collision energy (V)	Product 2 (m/z)	Collision energy (V)
Clonazepam	316	55	270	24	214	39

Results of analysis were validated statistically and also by recovery studies. The validation and critical parameters, calculated by using HPLC and UPLC–MS-MS for Clonazepam, are shown in Table 3.2.

Table 3.2.

Comparison of validation characteristics of HPLC and UPLC–MS-MS methods for clonazepam determination

Parameter	HPLC	UPLC–MS-MS
Pressure, bar	400	750
Volume of mobile phase, ml	40	21.5
Volume of sample injection, ml	20	5
Flow rate, ml/min	1	0.7
Retention time, min	15 ± 0.036	4.4 ± 0.017
Total run time, min	40	15
Linearity with R^2	> 0.991	> 0.996
Limit of detection, μg/ml	0.07	0.001

3.4. Economic evaluation of the proposed methods

According to the accounting regulations' standards, the costs included are direct labor costs, other direct costs, variable overhead, and fixed overhead [60]. Other expenses include the intra-factory movement of materials, semi-finished products, tools from warehouses to workshops, and finished products to warehouses and a lack of work in progress, payment for down-time, and so forth. The depreciation of fixed assets is a part of the overhead costs. It should be noted that the list and composition of items of cost products (works, services) are established by the enterprise independently. Therefore, considering the provisions of accounting regulations' standards on determining the cost of products (works, services) and the specificity of analytical research, we have formed the following cost items: basic raw materials, auxiliary materials, electricity, transport and procurement costs, wages, social security payments, fixed assets, recycling.The calculations were made

considering the time of analytical investigation for each method, namely HPLC analysis-40 min, UPLC–MS-MS -15 min, and the cost of the calculations are presented in Euro.

The cost of required reagents for analytical research was carried out based on the official company database of Sigma-Aldrich [61]. The procedure of the cost calculation is presented in Table 3.3.

Table 3.3.

Cost expenses under "basic raw materials and materials" and "supporting materials". Calculation unit – 1 sample analysis

Raw Material	Price, Euro	Quantity for Conducting 1 Test Sample	Cost, Euro
HPLC method			
The Main Raw Materials			
anhydrous dibasic ammonium phosphate, 100 g	22.10	0.66 g	0.15
phosphoric acid, 100 ml	165.00	6 ml	9.90
methanol, suitable for HPLC, ≥99.9%, 1l	45.30	104 ml	4.71
tetrahydrofuran, anhydrous, ≥99.9%, inhibitor-free, 1l	213.00	26 ml	5.54
water for chromatography 1L	31.90	60 mL	1.91
membrane filter 0,45 microns No. 100	250.0	1	2.50
vials, volume 2 mL, No. 100	23.50	1	0.24
Supporting Materials			
latex gloves with powder No. 100	12.56	1	0.13
disposable non-woven medical cap No. 100	2.50	1	0.03
shoe covers medical sterile No. 50	3.50	1	0.07
		Total	25.04

Continuation of table 3.3.

Raw Material	Price, Euro	Quantity for Conducting 1 Test Sample	Cost, Euro
UPLC–MS-MS method			
The Main Raw Materials			
methanol for UHPLC-MS LiChrosolv, 1 l	193.00	2 ml	0.39
di-Sodium tetraborate, anhydrous for analysis, 1 kg	188.00	1 mg	0.19
dichloromethane, HPLC Plus, for HPLC, GC, and residue analysis, ≥99.9%, contains 50-150 ppm amylene as stabilizer, 1 l	103.00	0.27 μL	0.01
ether, puriss., ≥99.9% (GC), 2 l	675.00	0.45 μL	0.01
hexane suitable for HPLC, ≥95%, 1 l	122.00	0.18 μL	0.01
water for chromatography 1 l	31.90	1 ml	0.03
Supporting Materials			
latex gloves with powder No. 100	12.56	1	0.13
disposable non-woven medical cap No. 100	2.50	1	0.03
shoe covers medical sterile No. 50	3.50	1	0.07
		Total	0.84

Costs such as wages, electricity, equipments and columns were not included in the cost, as these amounts vary from country to country and it is impossible to predict the level in advance.

However, Table 3.3 shows that the UPLC–MS-MS method is ten times more cost-effective than the liquid chromatography method suggested by the USP.

3.5. Eco-Scale Calculation

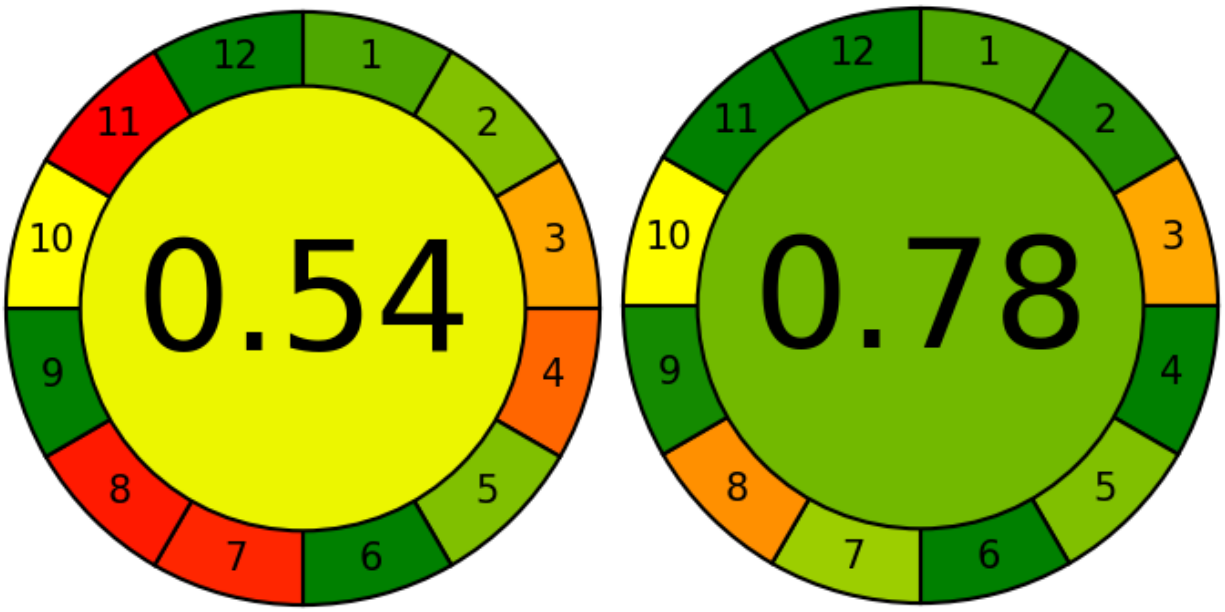
Chromatographic analysis requires the usage of various procedures and pre-treatment of analysed samples. Besides, for the assay of a sample, usually, a couple of determination methods are acceptable. The selection of analytical methods is commonly based on its accuracy, precision, cost, and the environmental and health impact [62].

Despite several reported approaches for determining greener nature of analytical procedures [63], only the “AGREE methodology” [64] employs all 12 green analytical chemistry principles:

1. Direct analytical techniques should be applied to avoid sample treatment.
2. Minimal sample size and minimal number of samples are goals.
3. In situ measurements should be performed.
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.
5. Automated and miniaturized methods should be selected.
6. Derivatization should be avoided.
7. Generation of a large volume of analytical waste should be avoided and proper management of analytical waste should be provided.
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.
9. The use of energy should be minimized.
10. Reagents obtained from renewable source should be preferred.
11. Toxic reagents should be eliminated or replaced.

12. The safety of the operator should be increased.

Accordingly, the greenness nature of the present approach was assessed utilizing “AGREE Calculator”. Figure 3.2 depicts the overall AGREE scale for the present analytical approach.



a b

Figure 3.2. Analytical GREEnness scale for the greener: a –HPLC, b – UPLC–MS-MS approach

Figure 3.2 lists the AGREE report sheet and AGREE score for each GAC principle. The overall AGREE scale for the proposed analytical methods HPLC, UPLC–MS-MS was calculated as 0.54, 0.78, respectively, indicating that the proposed UPLC–MS-MS method for the analysis of clonazepam is extremely green, but the HPLC method is not.

The ecological compatibility of the HPLC method was affected by the large number of reagents and volumes that would need to be disposed of, the analysis time (40 minutes), since only one analysis can be performed in 1 hour, and the lengthy multi-operational sample preparation. Therefore, in comparison with the HPLC method, it cannot be recommended as a green method.

CONCLUSION

1. To conduct the study, we considered the liquid chromatography method, which is approved by the US Pharmacopeia for the analysis of clonazepam in substance and finished medicinal products, and was also used to determine clonazepam in forensic materials, and the ultra-performance liquid chromatography–tandem mass spectrometry method, which is widely used in forensic and toxicological analysis due to the fact that it allows the determination of a substance in mixtures and biological fluids, including metabolites.

2. Based on the methods and results obtained, the UPLC–MS-MS method takes less time, requires less sample and has a sample limit of 0.001 μg/ml, but the HPLC method also meets the requirements for methods to be used in forensic analysis in all validation parameters.

3. As for the cost of analysis, the analysis of one sample of clonazepam by the HPLC method will cost EUR 25.04, and the UPLC–MS-MS method will cost EUR 0.84.

4. The overall AGREE scale to calculate the ecological indicator for the proposed analytical methods HPLC, UPLC–MS-MS was calculated as 0.54, 0.78, respectively, indicating that the proposed UPLC–MS-MS method for the analysis of clonazepam is extremely green, but the HPLC method is not.

GENERAL CONCLUSION

1. The study covers the indications for use, pharmacodynamics and pharmacokinetics of clonazepam, as well as cases of overdose and off-label use.

2. It has been determined that clonazepam is a widespread anxiolytic worldwide, which is one of the three drugs of the benzodiazepine group in terms of the frequency of court cases and forensic pharmaceutical and toxicological analyses.

3. Chromatographic methods for forensic pharmaceutical analysis of clonazepam in case files are considered. For each proposed methods, critical points are identified, including validation characteristics, cost and environmental friendliness.

4. Based on the methods and results obtained, the UPLC–MS-MS method takes less time, requires less sample and has a sample limit of 0.001 μg/ml, but the HPLC method also meets the requirements for methods to be used in forensic analysis in all validation parameters.

5. As for the cost of analysis, the analysis of one sample of clonazepam by the HPLC method will cost EUR 25.04, and the UPLC–MS-MS method will cost EUR 0.84.

6. The overall AGREE scale to calculate the ecological indicator for the proposed analytical methods HPLC, UPLC–MS-MS was calculated as 0.54, 0.78, respectively, indicating that the proposed UPLC–MS-MS method for the analysis of clonazepam is extremely green, but the HPLC method is not.

7. The UPLC–MS-MS method proved to be the optimal method for the forensic pharmaceutical analysis of clonazepam in terms of accuracy, environmental friendliness and cost-effectiveness. As for the HPLC method proposed by the US Pharmacopeia, it can also be used in laboratories for suitable tasks, as it meets all the requirements for methods set out in the Pharmacopoeias and legal legislation, but it requires more time for analysis and is more costly. In sum, the methods are interchangeable depending on the equipment of the laboratories.

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APPENDICES

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
НАЦІОНАЛЬНИЙ ФАРМАЦЕВТИЧНИЙ УНІВЕРСИТЕТ

СЕРТИФІКАТ УЧАСНИКА

Цим засвідчується, що

Benothmane R., Sych I.V.

Scientific supervisors: Bevz O.V., Perekhoda L.O.

брав(ла) участь у роботі

XXIX Міжнародної науково-практичної конференції молодих вчених та студентів

«АКТУАЛЬНІ ПИТАННЯ СТВОРЕННЯ НОВИХ ЛІКАРСЬКИХ ЗАСОБІВ»

В.о. ректора
Національного фармацевтичного університету — Алла КОТВІЦЬКА

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ * Національний фармацевтичний університет * №02010936 *

19-21 квітня 2023 р, м. Харків

APPENDIX A

APPENDIX A (cont.)

МІНІСТЕРСТВО ОХОРОНИ ЗДОРОВ'Я УКРАЇНИ
НАЦІОНАЛЬНИЙ ФАРМАЦЕВТИЧНИЙ УНІВЕРСИТЕТ

АКТУАЛЬНІ ПИТАННЯ СТВОРЕННЯ НОВИХ ЛІКАРСЬКИХ ЗАСОБІВ

МАТЕРІАЛИ
XXIX МІЖНАРОДНОЇ НАУКОВО-ПРАКТИЧНОЇ
КОНФЕРЕНЦІЇ МОЛОДИХ ВЧЕНИХ ТА СТУДЕНТІВ

19-21 квітня 2023 року
м. Харків

Харків
НФаУ
2023

APPENDIX A (cont.)

методикою Фармакопеї, без урахування вартості на електропостачання, заробітну плату персоналу та утилізацію викидів.

За аналітичною шкалою AGREE, методики є надзвичайно зеленими з числовими значеннями 0,77, 0,82 та 0,82 в середовищі метанолу, 0,1 М розчину кислоти хлористоводневої та 0,1 М розчину натрію гідроксиду, відповідно.

Висновки. Для ідентифікації міноксидилу в лікарських та косметичних засобах рекомендовано метод абсорбційної спектрофотометрії в інфрачервоній ділянці спектра, кількісне визначення проводити методом спектрофотометрії в ультрафіолетовій ділянці в середовищі 0,1 М розчину кислоти хлористоводневої, або 0,1 М розчину натрію гідроксиду, або метанолу. Через те, що обрані методики є експресними, екологічними, економічними, чутливими та специфічними.

THE CHOICE OF OPTIMAL METHODS OF DETERMINATION OF CLONAZEPAM FOR THE TASKS OF FORENSIC PHARMACEUTICAL ANALYSIS

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Introduction. Clonazepam, a benzodiazepine, is commonly used in treating various conditions, including anxiety disorders and epileptic seizures. Due to its low price and easy availability, however, it has become a commonly misused medication, both in medical and recreational contexts. Clonazepam, alone or in combination with other psychoactive substances, can lead to unwanted effects on health, such as motor and cognitive impairment, sleep disorders, and aggravation of mood and anxiety disorders. Prolonged use of clonazepam may lead to physical dependence and tolerance. Therefore, very often, the use of clonazepam leads to criminal acts. Therefore, it is often found in toxicological and forensic analysis in case files.

In this regard, it is important to select methods for the determination of clonazepam in substances, finished medicinal products, and mixtures in the presence of other components. It should be borne in mind that these methods must meet the requirements of the Pharmacopoeia for pharmaceutical analysis and the Ministry of Justice for the use of methods in forensic and toxicological analysis so that the results obtained have legal force and serve as evidence in court.

Therefore, the methods should be validated, and their choice should be justified by the following parameters: validation characteristics suitable for court cases, environmental friendliness, and cost-effectiveness.

The aim of the study. The aim of the study is to select a method for the determination of clonazepam for forensic pharmaceutical analysis, consideration of validation characteristics, material costs and environmental friendliness.

Materials and methods. compilation of data from reports on clonazepam analysis methods suitable for forensic pharmaceutical analysis, mathematical calculations and statistical processing of the results.

APPENDIX A (cont.)

Research results. To conduct the study, we considered the liquid chromatography (HPLC) method, which is approved by the US Pharmacopeia for the analysis of clonazepam in substance and finished medicinal products, and was also used to determine clonazepam in forensic materials, and the ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS-MS) method, which is widely used in forensic and toxicological analysis due to the fact that it allows the determination of a substance in mixtures and biological fluids, including metabolites.

The liquid chromatograph is equipped with a 254-nm detector and a 4.6-mm × 15-cm column that contains packing L7. Mobile phase – mixture of buffer solution (pH of 8.0), methanol, and tetrahydrofuran (60:52:13). The sample is diluted in a mixture of water, methanol, and tetrahydrofuran (60:52:13).

In ultra-performance liquid chromatography–tandem mass spectrometry, the sample is analyzed using a Waters Acquity UPLC and TQ detector using positive electrospray ionization and multiple reaction monitoring mode. The capillary voltage is 3 kV and the desolvation temperature is 400°C. The desolvation gas flow is set at 800 L/h and the source temperature is 120°C. An Acquity UPLC BEH C18 column with dimensions of 2.1 × 100 mm and a 1.7-µm particle size is used; column temperature is maintained at 50°C. The mobile phase flow rate was 0.40 mL/min, and the injection volume is 10 µL. The mobile phase is 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The sample is diluted in a mobile phase A.

The results of the analysis were validated statistically and also by recovery studies. The validation and critical parameters, calculated by using HPLC and UPLC–MS-MS for Clonazepam, are shown in Table 1.

Table 1. Comparison of validation characteristics of HPLC and UPLC–MS-MS methods for clonazepam determination

Parameter	HPLC	UPLC–MS-MS
Pressure, bar	400	750
Volume of mobile phase, ml	40	21.5
Volume of sample injection, ml	20	5
Flow rate, ml/min	1	0.7
Retention time, min	15 ± 0.036	4.4 ± 0.017
Total run time, min	40	15
Linearity with R^2	> 0.991	> 0.996
Limit of detection, µg/ml	0.07	0.001

Based on the methods and results obtained, the UPLC–MS-MS method takes less time, requires less sample and has a sample limit of 0.001 µg/ml, but the HPLC method also meets the requirements for methods to be used in forensic analysis in all validation parameters.

As for the cost of analysis, the analysis of one sample of clonazepam by the HPLC method will cost EUR 25.04, and the UPLC–MS-MS method will cost EUR 0.84.

The overall AGREE scale to calculate the ecological indicator for the proposed analytical methods HPLC, UPLC–MS-MS was calculated as 0.54, 0.78, respectively, indicating that the proposed UPLC–MS-MS method for the analysis of clonazepam is extremely green, but the HPLC method is not.

The ecological compatibility of the HPLC method was affected by the large number of reagents and volumes that would need to be disposed of, the analysis time (40 minutes), since only one analysis can be performed in 1 hour, and the lengthy multi-operational sample preparation. Therefore, in comparison with the HPLC method, it cannot be recommended as a green method.

APPENDIX A (cont.)

Conclusions. The UPLC–MS-MS method proved to be the optimal method for the forensic pharmaceutical analysis of clonazepam in terms of accuracy, environmental friendliness and cost-effectiveness. As for the HPLC method proposed by the US Pharmacopeia, it can also be used in laboratories for suitable tasks, as it meets all the requirements for methods set out in the Pharmacopoeias and legal legislation, but it requires more time for analysis and is more costly. In sum, the methods are interchangeable depending on the equipment of the laboratories.

USING PHARMACOPOEIAL METHODS FOR IDENTIFICATION AND ASSAY OF DOSAGE FORMS BASED ON CHLORHEXIDINE GLUCONATE

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Introduction. The use of antiseptics has increased significantly in various medical and professional settings, as well as in the home, due to their antiviral properties in the context of the ongoing coronavirus disease 2019 (COVID-19) pandemic. Also, the use of this class of drugs is widely used by consumers to prevent infection during seasonal colds and flu. In connection with such popularity of use, the aspect of studying the quality of this group of agents is very important, since the appearance of cheap analogues of many antiseptics in recent years indicates the use of low-quality substances or counterfeit products. Such actions can lead to undesirable side effects. Another aspect of poor quality, in addition to determining the authenticity of the active ingredients, is its quantitative content, which must correspond to that stated in the regulatory documentation or quality certificates. Failure to comply with this parameter can lead to a decrease in the antimicrobial and antiviral properties of the antiseptics used. Therefore, the study of existing and possible methods of quality control of known antiseptic agents is an urgent study.

Aim. Using of pharmacopoeial methods for the identification and assay of dosage forms based on Chlorhexidine gluconate in order to introduce potential quality control methods into the development.

Materials and methods. As objects, antiseptics based on Chlorhexidine gluconate (solution and gel) were chosen, which are widely used in dermatology, dentistry, surgery, as well as in everyday life for the treatment of wounds and skin disinfection. For the study, pharmacopoeias methods of analysis were used.

Results and discussion. For the identification and quantification (assay) of Chlorhexidine gluconate in dosage forms for topical use, the quality control methods of the European (Ph. Eur.) and American Pharmacopoeia (U.S.P.) monographs were used. The table systematizes the data of quality parameters for the solution and gel of Chlorhexidine gluconate.

Ф А 2.2.1–32-366

National University of Pharmacy

Faculty for foreign citizens' education
Department medicinal chemistry
Level of higher education master
Specialty 226 Pharmacy, industrial pharmacy
Educational program Pharmacy

APPROVED
The Head of Department
medicinal chemistry

Name SURNAME
"_22nd_" of August 2022

ASSIGNMENT
FOR QUALIFICATION WORK
OF AN APPLICANT FOR HIGHER EDUCATION

Reda BENOTHMANE

1. Topic of qualification work: «The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis», supervisor of qualification work: Olena BEVZ, PhD
approved by order of NUPh from "6th" of February 2023 № 35

2. Deadline for submission of qualification work by the applicant for higher education: April 2023.

3. Outgoing data for qualification work: statistics of poisonings, criminal cases and cases of falsification of medicinal products with benzodiazepine derivatives, including clonazepam, metabolism and mechanism of action of clonazepam, pharmacopoeial methods of clonazepam analysis.

4. Contents of the settlement and explanatory note (list of questions that need to be developed): to review the literature on the diseases for which clonazepam drugs are prescribed, their mechanism of action and pharmacological effects; to review statistical data on the use of clonazepam in the world, the frequency of falsification of medicines and off-label use, as well as forensic pharmaceutical analysis of case files; to review the physicochemical properties and modern methods of analysis of clonazepam; to select optimal methods for the determination of clonazepam for use in forensic pharmaceutical analysis; to calculate the cost of reagents, environmental friendliness and compare the validation characteristics for the proposed methods; to compare the results obtained and draw a conclusion about the optimal methods for the analysis of clonazepam in court cases

5. List of graphic material (with exact indication of the required drawings):

Tables – 4, pictures – 6, schemas – 1

6. Consultants of chapters of qualification work

Chapters	Name, SURNAME, position of consultant	Signature, date	
		assignment was issued	assignment was received
1	Olena BEVZ, assistant of department medicinal chemistry	September 2022	September 2022
2	Olena BEVZ, assistant of department medicinal chemistry	November 2022	November 2022
3	Olena BEVZ, assistant of department medicinal chemistry	January 2023	January 2023

7. Date of issue of the assignment: "_22nd_" of August 2022

CALENDAR PLAN

№ з/п	Name of stages of qualification work	Deadline for the stages of qualification work	Notes
1	Pharmacokinetics and pharmacodynamics, manifestations of long-term use and misuse of clonazepam. Writing 1 chapter.	Sept-Nov 2022	**done**
2	Study, processing and analysis of literature data on the use of clonazepam, methods of its synthesis, analysis and metabolism and physic-chemical properties. Writing 2 chapter.	Dec 2022 - Jan 2023	**done**
3	To choose of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis.	Jan-Feb 2023	**done**
4	Statistical processing of taken results. Writing 3 chapter.	March 2023	**done**
5	Summing up and submission to the Examination Board	April 2023	**done**

An applicant of higher education _____ Reda BENOTHMANE

Supervisor of qualification work _____ Olena BEVZ

ВИТЯГ З НАКАЗУ № 35
По Національному фармацевтичному університету
від 06 лютого 2023 року

нижченаведеним студентам 5-го курсу 2022-2023 навчального року, навчання за освітнім ступенем «магістр», галузь знань 22 охорона здоров'я, спеціальності 226 – фармація, промислова фармація, освітня програма – фармація, денна форма здобуття освіти (термін навчання 4 роки 10 місяців та 3 роки 10 місяців), які навчаються за контрактом, затвердити теми кваліфікаційних робіт:

Прізвище студента	Тема кваліфікаційної роботи		Посада, прізвище та ініціали керівника	Рецензент кваліфікаційної роботи
• **по кафедрі медичної хімії**				
Бенотман Реда	Підбір оптимальних методів визначення клоназепаму для завдань судово-фармацевтичного аналізу	The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis	асист Бевз О.В.	професор Георгіянц В.А.

Підстава: подання декана, згода ректора

Ректор

Вірно. Секретар

Ф А2.8-47-110

ВИСНОВОК

Комісії з академічної доброчесності про проведену експертизу
щодо академічного плагіату у кваліфікаційній роботі
здобувача вищої освіти

№ 112438 від « 21 » квітня 2023 р.

Проаналізувавши випускну кваліфікаційну роботу за магістерським рівнем здобувача вищої освіти денної форми навчання Бенотман Реда, 5 курсу, _____ групи, спеціальності 226 Фармація, промислова фармація, на тему: «Підбір оптимальних методів визначення клоназепаму для завдань судово-фармацевтичного аналізу / The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis», Комісія з академічної доброчесності дійшла висновку, що робота, представлена до Екзаменаційної комісії для захисту, виконана самостійно і не містить елементів академічного плагіату (компіляції).

Голова комісії,
професор **Інна ВЛАДИМИРОВА**

0%
33%

Ф А 2.2.1-32-353

REVIEW

of scientific supervisor for the qualification work of the master's level of higher education of the specialty 226 Pharmacy, industrial pharmacy

Reda BENOTHMANE

on the topic: «The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis»

Relevance of the topic. Forensic-pharmaceutical risks arise during the provision of medical care, circulation of medicines at the level of health care institutions (hospitals, pharmacies, polyclinics), pharmacotherapy accompanying health disorders, availability of all clinical-pharmacological, classification-legal and nomenclature- legal groups. One of the subjects of forensic pharmaceutical cases is clonazepam, which is seized and analyzed as a counterfeiting material and an off-label drug. All expenses for conducting a forensic analysis are borne by the state, therefore, the selection of economic and affordable methods is an urgent task, in addition, in order for the research results to have legal grounds, the methods used for the analysis must be pharmacopoeial and meet the requirements of international judicial law. Therefore, the discussed topic is relevant today.

Practical value of conclusions, recommendations and their validity. The practical value of the results of the qualification work consists in obtaining data of chromatographic methods, which can be considered in the future to justify the choice of one or another method of determining clonazepam in court cases.

Assessment of work. The work was performed at a high scientific level, the results obtained are reliable, the conclusions are logical and well-founded. The overall evaluation of the work is positive.

General conclusion and recommendations on admission to defend. The qualification work of Reda BENOTHMANE meets the requirements for qualification works in terms of the relevance and scope of the performed research,

the novelty of the obtained results, their theoretical and practical significance and can be recommended for defense at the Examination Commission.

Scientific supervisor _____ Olena BEVZ

« 7th» of April 2023

Ф А 2.2.1-32-356

REVIEW

for qualification work of the master's level of higher education, specialty 226 Pharmacy, industrial pharmacy

Reda BENOTHMANE

on the topic: «The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis»

Relevance of the topic. One of the main stages of conducting a forensic pharmaceutical analysis of case materials is the selection of optimal methods so that the results are subsequently considered in court. The correctness of the method must be confirmed by validation. In addition, the state now considers various economic and environmental factors. Therefore, the chosen methods of analysis that will be used in the judicial analysis must be reasoned. Since clonazepam often appears in the materials of court cases, studies conducted during the performance of qualification work are relevant.

Theoretical level of work. The qualification work was performed at a high theoretical level using modern theoretical approaches to the analysis of modern scientific literature and modern methods of analysis for conducting research for the substance to be determined.

Author's suggestions on the research topic. Modern pharmacopoeial methods of determining clonazepam in the materials of court cases are proposed. Validation characteristics, analysis costs and environmental friendliness are calculated for each method. The data confirm that, depending on the assigned tasks, the considered spectral and chromatographic methods can be used for forensic pharmaceutical examination of case materials suspected of containing clonazepam.

Practical value of conclusions, recommendations and their validity. The results obtained in the qualification work can be used when choosing methods for determining clonazepam, depending on the equipment of the laboratory and the availability of reagents.

Disadvantages of work. There are no fundamental comments regarding the content of the work, there are certain spelling errors that generally do not affect the content of the work.

General conclusion and assessment of the work. The qualification work of Reda BENOTHMANE in terms of relevance, scientific novelty of the obtained results, methodological level, theoretical and practical significance, volume of performed research meets the requirements of the Regulation on the Procedure for the Preparation and Defense of Qualification Works at the National Pharmaceutical University and can be recommended for defense at the Examination Commission.

Reviewer _____ prof. Viktoriia GEORGIANTS

«14th» of April 2023

Ф А2.2.1-91-287

ВИТЯГ
з протоколу засідання кафедри медичної хімії
№ 10 від 21 квітня 2023 р.

ПРИСУТНІ:

проф. Ліна ПЕРЕХОДА, проф. Андрій ФЕДОСОВ, доц. Вадим ЗУБКОВ, доц. Ірина СИЧ, доц. Віталій ЯРЕМЕНКО, доц. Ілля ПОДОЛЬСЬКИЙ, доц. Наталія КОБЗАР, доц. Марина РАХІМОВА, доц. Маргарита СУЛЕЙМАН, ас. Олена БЕВЗ, ас. Ольга ВІСЛОУС

ПОРЯДОК ДЕННИЙ:

Звіт про стан виконання кваліфікаційної роботи здобувача вищої освіти факультету з підготовки іноземних громадян Фм18(5,0д)англ-02 групи, спеціальності «226 Фармація, промислова фармація», освітньої програми «Фармація» Реди БЕНОТМАНА на тему: «Підбір оптимальних методів визначення клоназепаму для завдань судово-фармацевтичного аналізу / The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis»

СЛУХАЛИ: доповідь здобувача вищої освіти факультету з підготовки іноземних громадян Фм18(5,0д)англ-02 групи, спеціальності «226 Фармація, промислова фармація», освітньої програми «Фармація» Реди БЕНОТМАНА на тему: ««Підбір оптимальних методів визначення клоназепаму для завдань судово-фармацевтичного аналізу / The choice of optimal methods of determination of clonazepam for the tasks of forensic pharmaceutical analysis», керівник – асистент кафедри медичної хімії, к.фарм.н. Олена Бевз.

УХВАЛИЛИ: рекомендувати кваліфікаційну роботу Реди БЕНОТМАНА до офіційного захисту в Екзаменаційній комісії.

Завідувачка кафедри медичної хімії,
професор **Ліна ПЕРЕХОДА**

Секретар кафедри медичної хімії,
доцент **Марина РАХІМОВА**

Ф А2.2.1-32-042

НАЦІОНАЛЬНИЙ ФАРМАЦЕВТИЧНИЙ УНІВЕРСИТЕТ

ПОДАННЯ
ГОЛОВІ ЕКЗАМЕНАЦІЙНОЇ КОМІСІЇ
ЩОДО ЗАХИСТУ КВАЛІФІКАЦІЙНОЇ РОБОТИ

Направляється здобувач вищої освіти Реда БЕНОТМАН до захисту кваліфікаційної роботи
за галуззю знань 22 Охорона здоров'я
спеціальністю 226 Фармація, промислова фармація
освітньою програмою Фармація
на тему: «Підбір оптимальних методів визначення клоназепаму для завдань судово-фармацевтичного аналізу».

Кваліфікаційна робота і рецензія додаються.

Декан факультету _______ / Світлана КАЛАЙЧЕВА /

Висновок керівника кваліфікаційної роботи

Здобувач вищої освіти Реда БЕНОТМАН у повному обсязі виконала кваліфікаційну роботу. За актуальністю, методичним рівнем, теоретичним та практичним значенням, об'ємом виконаних досліджень кваліфікаційна робота відповідає вимогам і допускається до захисту в Екзаменаційній комісії.

Керівник кваліфікаційної роботи

_____ Олена БЕВЗ

«07» квітня 2023 р.

Висновок кафедри про кваліфікаційну роботу

Кваліфікаційну роботу розглянуто. Здобувач вищої освіти Реда БЕНОТМАН допускається до захисту даної кваліфікаційної роботи в Екзаменаційній комісії.

Завідувачка кафедри
медичної хімії

_____ Ліна ПЕРЕХОДА

«21» квітня 2023 р.

Qualification work was defended

of Examination commission on

« » of June 2022

With the grade _____

Head of the State Examination commission,

DPharmSc, Professor

_____ / Oleh SHPYCHAK /