RYNER FEGHALI

ANALYSIS OF PSYCHOTROPIC MEDICINES BROMAZEPAM, NITRAZEPAM AND TRIAZOLAM MIXTURE USING THIN-LAYER CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS

Master thesis

Thesis supervisor: Assoc. prof. Ph.D. Andrejus Ževžikovas

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Assoc. prof. PhD. Andrejus Ževžikovas __________________________
(Signature) (Date)

Reviewer

Master Thesis is accomplished by
Postgraduate Ryner Feghali

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SUMMARY

Master Thesis of R. Feghali “Analysis of psychotropic medicines bromazepam, nitrazepam and triazolam mixture using thin – layer chromatography and high-performance liquid chromatography methods“. Scientific supervisor Assoc. prof. Ph.D. Andrejus Ževžikovas; Lithuanian University of Health Sciences, Faculty of Pharmacy, Department of Analytical and Toxicological chemistry. – Kaunas

The aim of the thesis: To optimize thin-layer chromatography and high-performance liquid chromatography methods for bromazepam, nitrazepam, triazolam and their mixture qualitative analysis.

Objectives and methods: For optimization of TLC method bromazepam, nitrazepam, triazolam and their mixture solutions in methanol were analyzed. For mobile phase were used: cyclohexane, methanol, diethylamine, sulfuric acid, acetonitrile (solvents). For spots visualization was used: UV light lamp (254 nm; 365nm). Optimized methodic was applied for pharmaceutical products “Bromazepam Lannacher” (G.L. Pharma GmbH), “Eunoctin” (Gedeon Richter Plc.), “Halcion” (Pfizer H.C.P) (authorized in LT medicines) test solutions in cyclohexane-methanol-diethylamine (75:15:10) solvent system.

For optimization of HPLC method bromazepam, nitrazepam, triazolam and their mixture solutions of 0, 1 mg/ml in methanol were analyzed. Chromatograph with a photodiode array detector at wavelength 240 nm range and chromatographic column ACE C18 (5.0 cm x 2.1 mm) with sorbent particle size 5 μm were used for qualitative determination. The analysis was performed by using 0, 1 mol/L sulfuric acid-methanol – acetonitrile (7:8:9) mixture as eluent. Optimized methodic was applied in the analysis of pharmaceutical products “Bromazepam Lannacher” (G.L. Pharma GmbH), “Eunoctin” (Gedeon Richter Plc.), “Halcion” (Pfizer H.C.P) (authorized in LT medicines) test solutions in 0.1% sulphuric acid: ACN solvents.

Results. The best mobile phases for bromazepam, nitrazepam and triazolam mixture qualitative analysis using TLC was: “Cyclohexane-methanol-diethylamine (75:15:10) solvents system with an appropriate ratio. This mobile phase is suitable for examined substances separation, the qualitative analysis in mixture and determination in pharmaceutical products.
HPLC method was performed using ACE C18 (5.0 cm x 2.1 mm) chromatographic column. Gradient elution mode was used (a mixture of 0, 1% sulfuric acid buffer solvent and ACN solvent – as eluent). Flow rate 1 ml/min. Injection volume 10 μl. Photodiode array detector (200-600 nm wavelength). Bromazepam, nitrazepam, triazolam absorption of UV light was similar as in scientific literature. HPLC methodic is suitable for examined substances separation, the qualitative analysis in mixture and determination in pharmaceutical products.

**Conclusion.** TLC was confirmed to be suitable for the separation of bromazepam, nitrazepam, and triazolam pharmaceutical preparations based on the optimum conditions that were used for the analysis of “Bromazepam Lannacher” (G.L. Pharma GmbH), “Eunoctin” (Gedeon Richter Plc.), “Halcion” (Pfizer H.C.P) (authorized in LT medicines) test solutions. HPLC was also confirmed to be suitable for the separation of bromazepam, nitrazepam, and triazolam pharmaceutical based on the correlation with the reference solutions and analyzed (test) solution retention time which fell within the limits of the chosen HPLC method confidence interval. Therefore, the methods chosen were suitable for the qualitative assessment of the substances in medicines.
ABBREVIATIONS

AUC – Area under the curve

BZD – Benzodiazepine

Cmax - Peak plasma concentration

CNS – Central nervous system

DAD - Diode-Array Detector

DDD - Defined daily dose

GABA_A - Gamma-Aminobutyric acid

GI – Gastrointestinal

GS – Gas spectrometry

HPLC – High – performance liquid chromatography

ICU – Intensive care unit

IUPAC - International Union of Pure and Applied Chemistry

LCMS - Liquid chromatography-mass spectrometry

LOD – Limit of detection

LOQ - Limit of quantification

LT - Lithuania

MBNT - Mixture of bromazepam, nitrazepam, and triazolam

PNS – Peripheral nervous system

PVDF - Polyvinylidene fluoride
Rf – retention factor

RSB – Reference solution of bromazepam

RS MBNT – Reference solution of a mixture of bromazepam, nitrazepam, and triazolam

RSN – Reference solution of nitrazepam

RST – Reference solution of triazolam

RT – Retention time

SIL G – Silica gel

SS – Solvent system

TLC – Thin – layer chromatography

Tmax – Time to peak plasma concentration

UNODC - United Nations Office on Drugs and Crime

UV - Ultraviolet
INTRODUCTION

Benzodiazepine compounds are used for a variety of therapeutic indications, including anxiolytic, tranquilizer, and muscle relaxant, anticonvulsive, sedative or hypnotic. The pharmaceutical compounds belonging to this group are relatively safe when compared with barbiturates as they do not lead to a coma when used in high doses [1]. The inhibitory action of benzodiazepines on the central nervous system results from its interactions with the GABA\textsubscript{A} receptors, which are present in several brain regions [2]. Bromazepam, nitrazepam, and triazolam that were studied in this research are among important benzodiazepine derivatives used as anxiolytic, sedative or hypnotic drugs [1, 3, 4].

These facts lend importance to the study of the pharmacokinetics and bioavailability of these compounds and their concentrations in serum in cases of abuse: forensic cases, drug poisoning or suicidal excessive doses. These three components had not been included yet in the U.S. Pharmacopoeia till its 2010 edition [5]. The analytical methods described by the British, European and Japanese Pharmacopoeia in the monographs of the three studied compounds depend on the anhydrous titration by perchloric acid in the presence of acetic anhydride, and the endpoint is determined by a potentiometer [3,6,7]. Literature reviews have listed a number of publications on the analysis and determination of therapeutic and toxic blood concentrations of bromazepam, nitrazepam, and triazolam either as raw materials or in serum [8]. But the analysis of the mixture, as in this research, was not found in any of these publications [9]. These researches have adopted several methods, including HPLC [9], LCMS [10], in addition to electrochemical and spectral methods. The aim of this study was to develop a valid, rapid and sensitive analytical procedure using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) for the qualitative evaluation and mixture separation of bromazepam, nitrazepam, and triazolam.
AIM AND WORK TASKS

Aim: To implement optimization of thin-layer chromatography and high-performance liquid chromatography method for bromazepam, nitrazepam, and triazolam and their mixture qualitative analysis.

Tasks:

1) To optimize the TLC methodic which will be suitable for bromazepam, nitrazepam and triazolam and their mixture qualitative evaluation and mixture separation.

2) To optimize the HPLC methodic which will be suitable for bromazepam, nitrazepam and triazolam and their mixture qualitative evaluation and mixture separation.

3) To adapt the optimized TLC and HPLC methodics for bromazepam, nitrazepam and triazolam excreted from medicines for qualitative evaluation.

4) Validation of suitable HPLC methodic for its usage in quantitative analysis of investigated pharmaceuticals.
1. LITERATURE REVIEW

1.1. Bromazepam, Nitrazepam and Triazolam prevalence of use.

1.1.1. Bromazepam, Nitrazepam and Triazolam prevalence of use in Lithuania and Baltic countries.

Based on the Baltic Statistic Medicines report [11] in the Baltic countries (Estonia, Latvia, and Lithuania) the consumption of anxiolytics during 2013-2015 had a tendency of augmentation by DDD/1000/day. Lithuania has the highest consumption among the three countries.

![Consumption of anxiolytics (N05B)](image)

**Fig. 1 Consumption of anxiolytics in Baltic countries in the year 2013, 2014, 2015 per DDD/1000/day.**

Anxiolytics consumption was 41.43 DDD/1000/day in 2013, has increased to 41, 65 DDD/1000/day in 2014, and consequently decreased 41, 01 DDD/1000/day in 2015 in Lithuania, however, the consumption among the Baltic countries, clearly shows that from 2013 up to 2015 there is correlation in usage of bromazepam among the three countries, although Lithuania remains stable the country with significant increase and the most usage of bromazepam [11, 12].
Table 1. Consumption of Bromazepam in 2013, 2014, 2015 per DDD/1000/day in Baltic countries.

<table>
<thead>
<tr>
<th>Bromazepam</th>
<th>DDD/1000/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2013</td>
</tr>
<tr>
<td>Estonia</td>
<td>1.44</td>
</tr>
<tr>
<td>Latvia</td>
<td>2.82</td>
</tr>
<tr>
<td>Lithuania</td>
<td>6.07</td>
</tr>
</tbody>
</table>

It has been reported that usage of bromazepam by 2017 has reached 6.36.

Consumption of hypnotics and sedatives (N05C)

Fig. 2 Consumption of hypnotics and sedatives in Baltic countries in the year 2013, 2014, 2015 per DDD/1000/day.

Usage of hypnotic and sedatives benzodiazepine derivate in Lithuania accounted for 6.90 DDD/1000/day in 2013, 7.38 DDD/1000/day in 2014, consequently reaching up to 8.29 DDD/1000/day in 2015 [11].
Table 2. Consumption of Nitrazepam in 2013, 2014, 2015 per DDD/1000/day in Baltic countries.

<table>
<thead>
<tr>
<th>Nitrazepam</th>
<th>DDD/1000/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2013</td>
</tr>
<tr>
<td>Estonia</td>
<td>0.90</td>
</tr>
<tr>
<td>Latvia</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lithuania</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Table 3. Consumption of Triazolam 2013, 2014, 2015 per DDD/1000/day in Baltic countries.

<table>
<thead>
<tr>
<th>Triazolam</th>
<th>DDD/1000/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2013</td>
</tr>
<tr>
<td>Estonia</td>
<td>0.05</td>
</tr>
<tr>
<td>Latvia</td>
<td>0.15</td>
</tr>
<tr>
<td>Lithuania</td>
<td>1.14</td>
</tr>
</tbody>
</table>

It has been reported that usage of nitrazepam in 2017 has decreased to 0.45 DDD/1000/day. Since 2013, which has been shown to be the highest amount of consumption of nitrazepam for the last couple of years, however, triazolam in 2016 shown increase reaching up to 2,235 DDD/100/day, which is almost two times higher than in 2013, but by 2017 amount has decreased to 1.65. According to data, Lithuania remains the leading country in consumption of bromazepam and triazolam, on another hand, Estonia shows the greatest report in the usage of hypnotics and sedatives including nitrazepam [11, 12].

Table 4. Consumption of Bromazepam, Nitrazepam, and 2013-2017 per DDD/1000/day in Lithuania.
## 1.1.2. Bromazepam, Nitrazepam and Triazolam prevalence of use in other countries

In 2013, the global calculated consumption for this group of benzodiazepines reached 21.8 billion S-DDD (fig.3). The United States, Brazil, Canada, France, Spain, Argentina, and Italy (in descending order) were the largest consumers of this group of benzodiazepines, in absolute terms, in 2013. The calculated consumption of benzodiazepine-type anxiolytics is in Uruguay (634 S-DDD), Finland (280 S-DDD), Ireland (230 S-DDD), Canada (94 S-DDD), Portugal (88 S-DDD), Hungary (80 S-DDD) and Argentina (63 S-DDD) having the highest rates. Until 2010, Europe was systematically the region with the highest calculated average of national consumption rates for benzodiazepine-type anxiolytics (fig. 4) [13].

<table>
<thead>
<tr>
<th>Name of pharmaceutical</th>
<th>DDD/1000/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2013</td>
</tr>
<tr>
<td>Bromazepam</td>
<td>6,071</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>0,574</td>
</tr>
<tr>
<td>Triazolam</td>
<td>1,14</td>
</tr>
</tbody>
</table>
Fig. 3. Benzodiazepine-type anxiolytics: calculated global consumption\textsuperscript{a} 2004-2013

\textsuperscript{a}Statistical data submitted by Governments are used to calculate average annual consumption for a three-year period, expressed in S-DDD.
\textsuperscript{b}The data for 2013 are incomplete.
Global calculated consumption of bromazepam, which had been quite stable during the period 2004-2013, averaging 1.3 billion S-DDD per year, increased to 1.5 billion S-DDD in 2013, the largest amount recorded in the last ten years. The Democratic Republic of the Congo (276 million S-DDD), Brazil (204 million S-DDD), France (199 million S-DDD), Pakistan (116 million S-DDD) and Serbia (79 million S-DDD) had the highest levels [13].

Calculated global consumption of benzodiazepine-type sedative-hypnotics was less variable, varying around an annual average of 8 billion S-DDD during the period 2004-2013. In 2013, calculated global consumption stood at 7.1 billion S-DDD (see figure 5).
Up to 2010, the calculated average annual consumption of benzodiazepine-type sedative-hypnotics, expressed in S-DDD per 1,000 inhabitants per day, was traditionally higher in Europe and in a few non-European countries, such as Japan and Cuba, than in other regions. In recent years, however, there have been significant changes in average annual consumption rates across regions (see figure 6). While average consumption rates in Oceania have been relatively stable in the past six years, at about 9 S-DDD per 1,000 inhabitants per day, consumption rates in Europe have shown a downward trend, from 25 S-DDD at the beginning of the decade, to 17.5 S-DDD. At the same time, consumption rates have steadily increased in Asia, from 16 S-DDD to 18 S-DDD, and in Africa, albeit at a low level (from 1 S-DDD to 2 S-DDD). In 2013, Belgium, Japan, Cuba, Spain, and Italy, in that order, had the highest rates of consumption globally, with 66.7 S-
DDD, 46.5 S-DDD, 33 S-DDD, 28.6 S-DDD and 25.2 S-DDD per 1,000 inhabitants per day, respectively [13].

![Graph showing average national consumption of benzodiazepine-type sedative-hypnotics by region, 2005-2013.](image)

*Statistical data submitted by Governments are used to calculate the average annual consumption for a three-year period.*

**Fig. 6. Benzodiazepine-type sedative-hypnotics: average national consumption, a by region, 2005-2013**

Global calculated consumption of nitrazepam, which averaged over 6 tons (1.2 billion S-DDD) annually during the period 2004-2010, significantly declined in 2011 and dropped to 3.1 tons (623 million S-DDD) in 2013, the lowest level in the last ten years. Cuba, Norway, Malta, Japan, Ireland, and Croatia, in descending order, had the highest rates of calculated consumption in 2013.

Total calculated consumption of triazolam held at 988 million S-DDD in 2013, with the United States (570 million S-DDD), Japan (266 million S-DDD), Italy (97 million S-DDD) and Austria (22 million S-DDD) being the main consumers in absolute terms. Austria (7.2 S-DDD),
Japan (5.8 S-DDD), the United States (5 S-DDD) and Italy (4.4 S-DDD) had the highest calculated consumption rates of triazolam [13].

1.2. Statistic of poisoning using Bromazepam, Nitrazepam, Triazolam

Presently, benzodiazepines are among the most extensively used group of drugs worldwide because of their multiple therapeutic actions as anxiolytics, sedative, and hypnotics. The occurrence of the benzodiazepine dependence and withdrawal symptoms is directly dependant on the duration of drug use. Though they are considered safe and are commonly well-tolerated, there is a significant risk of misuse and abuse of benzodiazepines.

Benzodiazepine poisoning, specifically bromazepam, nitrazepam, triazolam may happen due to excessive consumption of the drug not in the right dosage than prescribed, however, its really rare for it to lead to a fatal case, unless high doses of benzodiazepine were taken in combination with alcohol, barbiturates, opioids or tricyclic antidepressants. Thus, it may lead to severe complications such as coma or death.

Most frequent symptoms of misuse or abuse of benzodiazepines are dizziness, confusion, drowsiness, blurred vision, unresponsiveness, anxiety, and agitation. Oral benzodiazepine (BZD) overdoses, without co-ingestions, rarely result in significant morbidity (e.g., aspiration pneumonia, rhabdomyolysis) or mortality. Acute intravenous administration of BZDs is associated with greater degrees of respiratory depression [14].

As BZD is one of the class of medication, which is commonly prescribed, it shows the very high amount of cases of self-poisoning due to a different reason, one of them inappropriate dosage intake. During 1996 and 2013, the number of adults filling a benzodiazepine prescription increased up to 67%, from 8.1 million to 13.5 million, as well as the rate of overdose deaths including benzodiazepines grew more than 4-fold from 0.58 to 3.07 per 100 000 adults; nevertheless, this rate appeared to plateau after 2010 [15].

1.3. Bromazepam, Nitrazepam, Triazolam toxicity
Benzodiazepines have a wide therapeutic index and may rarely cause severe complications or lethal case while taken alone in overdose. Usually, a patient who accidentally takes more than prescribed dosage will just feel somnolent and fall asleep for few hours.

The various benzodiazepines, to which belong bromazepam, nitrazepam, triazolam, differ in their toxicity since they produce varying levels of sedation in overdose, as well as having variable potency, duration effect, presence or absence of active metabolite and therapeutic indication. As for the pharmaceuticals in this work were taken in consideration, bromazepam half-life – 10-20 (h) with an active metabolite 3-hydroxybromazepam, which has a half-life approximately equal to that of bromazepam, and an oral dosage that range between 1.5-6 mg, triazolam has a half-life - 1.5-5.5 hours, with no active metabolite and an oral dose that range between 0.125-0.25 mg, nitrazepam half-life – 15-38 (h) with no active metabolites and an oral dose that ranges between 5-10 mg, but largely bound to plasma proteins (70%) [16].

Nitrazepam is a drug which is very frequently involved in drug intoxication, including overdose. Nitrazepam overdose may result in stereotypical symptoms of benzodiazepine overdose including intoxication, impaired balance, and slurred speech. In cases of a severe overdose, this may progress to a comatose state with the possibility of death.

1.3.1. Mechanism of toxicity

Benzodiazepines work by enforcing the activity of gamma-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the central nervous system (CNS). BZDs bind to a specific receptor on the GABAA receptor complex and in this way contribute the binding of GABA to its specific receptor site. BZD binding induces enhanced the frequency of opening of the chloride channel complexed with the GABAA receptor. Chloride channel opening results in membrane hyperpolarization, which inhibits cellular excitation.

Enhanced GABA neurotransmission results in sedation, striated muscle relaxation and anxiolysis. Stimulation of peripheral nervous system (PNS) GABA receptors may cause decreased cardiac contractility and vasodilation. These changes could have the potential to alter tissue perfusion [14].
Benzodiazepines enhance the action of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). They also inhibit other neuronal systems by poorly defined mechanisms. The result is generalized depression of spinal reflexes and the reticular activating system. This can cause coma and respiratory arrest. Respiratory arrest is more likely with newer short-acting benzodiazepines such as triazolam.

1.3.2. Toxic dosage

In general, the toxic-therapeutic ratio for benzodiazepines is very high. However, a respiratory arrest has been reported after ingestion of 5 mg of triazolam [17]. It is short-acting hypnotics and relatively safe in low prescribed doses, but there are a few reports of the fatal cases. Kinoshita et al. [8] have described the case where the patient has swallowed approximately 4.9 mg of triazolam. In that case, however, the ingested amount of triazolam may have been larger than the estimated amount, because there was a large amount of unabsorbed drug left in his stomach. The total ingested dose of triazolam was the sum of the above value and the dose left in the stomach. Thus, it was estimated that he had ingested at least 6.7 mg of triazolam. From the autopsy findings and the results of the toxicological examination, it was concluded that the victim died due to an overdose of triazolam, soonly after ingestion [18]. Also, ingestion of another drug with CNS-depressant properties (e.g., ethanol, barbiturates, and opioids) probably will produce additive effects [17]. As well as there were presented fatal cases of overdose of nitrazepam, but due to simultaneous consumption of alcohol and other psychotropic medications [19].

1.3.3. Treatment and antidote for an overdose of benzodiazepines

As with any overdose, the first step is an assessment of the patient's airway, breathing, and circulation, and these should be assessed as fast is it possible. In any patient with an altered mental status, a blood glucose level should be measured instantly. The foundation of treatment in BZD overdoses is good supportive care and monitoring.

Single-dose activated charcoal is not usually recommended as the risks far overbalance the benefit. BZDs overdose is very rarely fatal, although the resulting altered mental status greatly increases the risk of aspiration following oral charcoal dose [14].
Flumazenil is a competitive BZD receptor antagonist and is the only available specific antidote for BZDs. Its use in acute BZD is controversial, nevertheless, and its risks usually outweigh any benefit [20]. Common adverse reaction with flumazenil includes agitation and gastrointestinal symptoms, while serious adverse events include supraventricular arrhythmia and convulsions [21].

Flumazenil does not consistently reverse central respiratory depression due to BZDs, and over half the patients in one study experienced re-sedation after use. In long-term BZD users, flumazenil may lead to withdrawal and seizures; in patients taking BZDs for a medical condition, flumazenil may result in exacerbation of the condition [21].

Moreover, to those patients on long-term of BZD use, flumazenil should not be used in any patient at an increased risk of having a seizure, including those with a seizure history, head injury, co-ingestion of BZD and tricyclic antidepressant or other proconvulsant, or even a possible ingestion of a proconvulsant [22].

The ideal consideration for flumazenil use is for isolated iatrogenic BZD overdose in BZD-naive patients (e.g., during conscious sedation of a BZD-naive patient).

Patients may be discharged if they stay asymptomatic at least 6 hours after ingestion. Those with mild toxicity may be supervised in the emergency department until they recover. Patients with intentional overdoses demand a psychiatric evaluation before discharge. Admit patients with hemodynamic instability, coma, or respiratory depression to the intensive care unit (ICU). Respiratory depression may be cured with assisted ventilation [14].

1.4. Bromazepam

Bromazepam is a benzodiazepine derivative drug. It is mainly used for the short-term relief of symptoms of excessive anxiety and panic states. It works to reduce anxiety by affecting certain substances in the brain called neurotransmitters.
IUPAC formula: 7-bromo-5-pyridin-2-yl-1, 3-dihydro-1, 4-benzodiazepin-2-one

Chemical - physical characteristics, solubility and etc.

Bromazepam is white or yellowish, crystalline powder. Practically insoluble in water, slightly soluble or sparingly soluble in ethanol (96 percent) and in methylene chloride [6].

Pharmacological properties

Bromazepam is a lipophilic, long-acting benzodiazepine and with a sedative, hypnotic, anxiolytic and skeletal muscle relaxant properties. It does not possess any antidepressant qualities. Bromazepam shares with other benzodiazepines the risk of abuse, misuse, psychological and/or physical dependence. According to the literature, bromazepam has greater abuse potential rather than other benzodiazepines because of fast absorption and rapid onset of action [23].

Pharmacokinetics

Bromazepam, taken in the fasting state, is almost completely absorbed. Peak plasma levels of bromazepam are achieved between 0.5 – 4 hours and may be endorsed for up to 12 hours. The mean peak bromazepam level after a 12 mg oral dose is about 140 ng/mL.

On average, 70% of bromazepam is bound to plasma proteins with the volume of distribution is about 50 L. Bromazepam undergoes extensive metabolism. The main metabolic pathway includes hydroxylation in position 3 with subsequent glucuronidation and cleavage of the heterocyclic ring with subsequent hydroxylation in the benzene ring and conjugation. Two metabolites predominate 3-hydroxy-bromazepam and 2-(2-amino-5-bromo-3-hydroxy benzoyl) pyridine.

Less than 2% of a dose is excreted unchanged. The urinary recovery of intact bromazepam and the glucuronide conjugates of 3-hydroxy-bromazepam and 2-(2-amino-5-bromo-3-hydroxy benzoyl) pyridine is 2%, 27% and 40% of the administered dose. Bromazepam
has an elimination half-life of about 17 h (range 11 – 22 h). The clearance is about 40 mL/min [24].

1.5. Nitrazepam

Nitrazepam is a hypnotic drug of the benzodiazepine class used for short-term relief of anxiety and insomnia [25].

IUPAC formula: 7-nitro-5-phenyl-1, 3-dihydro-1, 4-benzodiazepin-2-one.

Chemical-physical characteristics, solubility and etc.

Nitrazepam is a yellow, crystalline powder; odorless or almost odorless. Practically insoluble in water; slightly soluble in ethanol; very slightly soluble in ether [6].

Pharmacological properties

Nitrazepam is a type of benzodiazepine drug. It is a powerful hypnotic drug which possesses strong sedative, anxiolytic, amnestic, anticonvulsant, and skeletal muscle relaxant properties. Nitrazepam shortens the time required to fall asleep and lengthens the duration of sleep [26].

Pharmacokinetics

Nitrazepam is well and fairly rapidly absorbed from the GI tract. Time to reach peak plasma concentrations following oral administration is about 2 hours with a range of 0.5 - 5 hours. Nitrazepam is lipophilic and readily crosses body membranes. Nitrazepam is approximately 87% bound to plasma protein. The major pathway is conversion to 7-aminonitrazepam and then to 7-acetamido-nitrazepam with subsequent hydroxylation. The opening of the diazepine ring to form 2-amino-5-nitrobenzophenone has also been reported. These metabolites have very weak pharmacological activity.
Nitrazepam is mainly excreted as urinary metabolites. During the first 120 hours after a single radio-labeled 10 mg oral dose, the total renal elimination was 70%. Only 1% or less of the administered dose is excreted as unchanged nitrazepam. The main urinary excretion products are free or conjugated 7-amino nitrazepam and 7-acetamido nitrazepam. Individual variation of the total excreted metabolites is high, ranging between 17 and 99% of the administered dose. Of this, the conjugated metabolites made up an average of 57% [27].

1.6. Triazolam

Triazolam is a benzodiazepine hypnotic with a very short elimination half-life (about 3 hours).

IUPAC formula: 8-chloro-6-(2-chlorophenyl)-1-methyl-4H-[1,2,4]triazolo[4,3a][1,4]benzodiazepine.

Chemical-physical characteristics, solubility and etc.

Triazolam is a white to off-white, practically odorless, crystalline powder. It is soluble in chloroform; slightly soluble in alcohol; practically insoluble in ether and in water [28].

Pharmacological properties

Triazolam is indicated for the symptomatic relief of transient and short-term insomnia in patients who have difficulty falling asleep. Triazolam is not recommended for early morning awakenings. Treatment with triazolam should usually not exceed 7-10 consecutive days. Use for more than 2-3 consecutive weeks requires a complete reevaluation of the patient. The use of hypnotics should be restricted for insomnia where disturbed sleep results in impaired daytime functioning [28].

Pharmacokinetics

Triazolam is rapidly absorbed and peak plasma levels are reached within 2 hours following oral administration. Peak plasma concentration (Cmax) and area under the plasma
concentration curve (AUC) increase in proportion to the dose, while the time to peak plasma concentration (Tmax), elimination half-life (t1/2β), and clearance are independent of dose. Triazolam has a short half-life; the range is reported to be 1.5 to 5.5 hours.

Triazolam is metabolized via hepatic microsomal oxidation. The hydroxylated metabolites, which are inactive, are excreted primarily in the urine as conjugated glucuronides. The two primary metabolites account for approximately 80% of urinary excretion. Repeated administration of triazolam for 7 days does not lead to accumulation and does not alter the rate of elimination [28].

1.7. Bromazepam, nitrazepam and triazolam qualitative evaluation using thin-layer chromatography (TLC) method.

1.7.1. Thin-layer chromatography method (TLC)

Thin layer chromatography is a technique of separation in which a mobile phase consisting of an appropriate solvent is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solution of analyzed products are applied on the plate prior to development, the separation is based on such factors like absorption, partition, ion exchange or combinations of these mechanisms and is carried out by movement of solutes in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase) [6].

The apparatus is consisting of a plate, a chromatographic tank, micropipette, fluorescence detection device, visualization devices and reagents, documentation.

Thin – layer liquid chromatography among various chromatographic methods is widely used for the separation of a particular benzodiazepine from its impurities or related compounds, as well as for screening of BZD and their metabolites. TLC methodic is recommended for identification of a large number of substances, which allows noticing the substance in a relatively small amount of time, therefore accounts to be a fast technique for comparative studies. TLC is mostly used for the rapid preference in pharmaceutical analysis because of a number of advantages that are seen: it simplicity, accuracy, cost-effectiveness and the possibility of simultaneous determination of a number of samples on a single TLC plate. However, it may
provide some differential data results from the other chromatographic method, which is HPLC for example and can give values higher than in HPLC. Benzodiazepines form a group of diverse chemicals, but the use of a combination of the TLC systems listed below provides good separation [29, 30].

1.7.2. Thin-layer-chromatography methods suitable for bromazepam, nitrazepam, and triazolam identification

The scientific literature contains various analysis by TLC method which may be used for bromazepam, nitrazepam and triazolam identification in medicinal fluids or pharmaceutical preparations. Most of the analytical methods are adapted to analyze individually or having a close chemical structure and physicochemical properties of the compounds – mostly medicinal substances such as benzodiazepine compounds or opioid analgesics. Table 5, 6, 7 provide information on the literature for thin layer chromatography techniques, which can be applied for quality assessment of bromazepam, nitrazepam, and triazolam.

There were identified a multiple of methods in the literature suitable for determination of bromazepam, nitrazepam, and triazolam. However, the following thin-layer chromatographic systems applied to benzodiazepines, when used in combination, give good separations for it.

According to UNODC “Recommended methods for the identification and analysis of barbiturates and benzodiazepines” [31]. Three suitable systems were:

Table 5. Rf index for bromazepam, nitrazepam, triazolam according to UNODC methods.

<table>
<thead>
<tr>
<th>System</th>
<th>Solvent system</th>
<th>Bromazepam Rf values</th>
<th>Nitrazepam Rf values</th>
<th>Triazolam Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Chloroform - acetone (80:20, v/v)</td>
<td>0,03</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>B</td>
<td>Chloroform - methanol (90:10, v/v)</td>
<td>0,13</td>
<td>0,42</td>
<td>0,09</td>
</tr>
<tr>
<td>C</td>
<td>Cyclohexane – toluene - diethylamine (75:15:10, v/v/v)</td>
<td>0,55</td>
<td>0,64</td>
<td>0,52</td>
</tr>
</tbody>
</table>
According to UNODC methods for the solvent system chosen, it has been applied 2μl of a 5mg/ml sol in methanol for determination. The chambers were saturated, stationary phase was silica gel, or silica gel impregnated with 0.1 mol/L KOH in methanol (system B and C) and dried prior to analysis. Visualization was obtained with UV light at 254nm or with help of a location reagent 2N H₂SO₄/heat/observe under UV light at 366 nm, and acidified potassium iodoplatinate reagent [31].

*Table 6. Rf index for bromazepam, nitrazepam, triazolam according to Clark’s analysis [32].*

<table>
<thead>
<tr>
<th>System</th>
<th>Solvent system</th>
<th>Bromazepam Rf values</th>
<th>Nitrazepam Rf values</th>
<th>Triazolam Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>Methanol – 25% ammonia (100:1,5)</td>
<td>0,61</td>
<td>0,68</td>
<td>0,6</td>
</tr>
<tr>
<td>TB</td>
<td>Cyclohexane – toluene – diethylamine (15:3:2)</td>
<td>0,06</td>
<td>-</td>
<td>0,01</td>
</tr>
<tr>
<td>TC</td>
<td>Chloroform – methanol (9:1)</td>
<td>0,41</td>
<td>0,36</td>
<td>0,4</td>
</tr>
<tr>
<td>TD</td>
<td>Chloroform – acetone (4:1)</td>
<td>0,13</td>
<td>0,35</td>
<td>0,05</td>
</tr>
<tr>
<td>TE</td>
<td>Ethyl acetate – methanol – 25% ammonia (17:2:1)</td>
<td>0,63</td>
<td>0,64</td>
<td>0,44</td>
</tr>
<tr>
<td>TF</td>
<td>Ethyl acetate</td>
<td>-</td>
<td>0,46</td>
<td>0,02</td>
</tr>
<tr>
<td>TL</td>
<td>Acetone</td>
<td>0,53</td>
<td>0,55</td>
<td>0,16</td>
</tr>
<tr>
<td>TAD</td>
<td>Chloroform–methanol (9:1)</td>
<td>0,47</td>
<td>0,53</td>
<td>0,41</td>
</tr>
<tr>
<td>TAE</td>
<td>Methanol</td>
<td>0,73</td>
<td>0,84</td>
<td>0,68</td>
</tr>
<tr>
<td>TAF</td>
<td>Methanol – n-butanol (3:2) containing 0.1mol/L NaBr</td>
<td>0,69</td>
<td>0,86</td>
<td>0,65</td>
</tr>
</tbody>
</table>
According to Clark’s analysis [32], it has been applied 2μl of a 2mg/ml sol in methanol for determination. The chambers were saturated, stationary phase was silica gel, or silica gel impregnated with 0.1 mol/L KOH in methanol and dried prior to analysis. Visualization was performed with UV light (both 254 and 350 nm). There are a broad variety of location reagents used: Location reagents for systems TA, TB and TC: Ninhydrin spray violet pink spots appear, FPN reagent blue spots appear, Dragendorff – yellow, orange or brown orange spots, acidified iodoplatinate solution giving violet, blue-violet colored spots. Location reagents for systems TD, TE, and TF: Van Urk’s reagent, Ferric chloride, Mercurous nitrate, Acidified potassium permanganate, Furfuraldehyde reagent, Acidified iodoplatinate solution. Location reagents for systems TAJ, TAK, TAL: cupric chloride, Dragendorff’s spray, Fearon’s reagent, Ferric chloride, ethanol and sulfuric acid, fluorescamine, Gibb’s reagent, concentrated hydrochloric and ethanol, iodine, Mandelin’s reagent, modified Ehrlich’s reagent, sodium nitrite, ninhydin.

**Table 7. Rf index for bromazepam, nitrazepam, triazolam according to Hancu et al [33].**

<table>
<thead>
<tr>
<th>System</th>
<th>Solvent system</th>
<th>Bromazepam Rf values</th>
<th>Nitrazepam Rf values</th>
<th>Triazolam Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ethyl acetate</td>
<td>0,42</td>
<td>0,77</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Chloroform – methanol (9:1)</td>
<td>0,48</td>
<td>0,75</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>Chloroform – acetone (4:1)</td>
<td>0,22</td>
<td>0,45</td>
<td>-</td>
</tr>
</tbody>
</table>
According to Hancu et al. [33], the BZD samples were prepared at a concentration of 0.5% in methanol; amounts of 0.5 µl were spotted on the chromatoplate. The chromatographic chambers were saturated with the mobile phase for 30 minutes. The plates were developed over a distance of 15 cm, dried in a stream of hot air, and examined first under UV radiation at wavelengths of 254 and 366 nm.

The tables show that most of the solvent systems described are not suitable for bromazepam, nitrazepam and triazolam mixture separation. Frequently, in case of separation, it is not possible because of Rf values which are really close to each other or solvent system adequacy has not been evaluated for all three materials. As TLC mobile phase components often used chloroform, acetone, methanol, ammonium hydroxide, ethyl acetate, cyclohexane, toluene, and diethylamine. While analyzing the literature it has not been found any information about bromazepam, nitrazepam and triazolam mixture qualitative assessment by TLC, and it was concluded that the optimal mixture testing mobile phase will be sought experimentally.

1.8. Bromazepam, nitrazepam and triazolam qualitative evaluation using high-performance liquid chromatography (HPLC) method

1.8.1. High-performance liquid chromatography method (HPLC)

Liquid chromatography is a method of chromatographic separation based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid which moves through a mobile phase contained in a column. Liquid
HPLC is used for the qualitative and quantitative determination of benzodiazepines. The importance of this method for screening is contradictory due to the reproducibility of retention times from column to column and dependence on some factors. The analysis of benzodiazepines in biological samples uses reversed-phase columns [34].

The mobile phases are acidic, a mixture of phosphate buffer and acetonitrile and/or methanol. It is important to control the pH of the mobile phase because even a small difference significantly affects the results of chromatography. For complex mixtures of benzodiazepines or in the analysis of the unknown, a gradient analysis or a combination of isocratic systems is necessary. Most benzodiazepines are determined at 230 nm, nitro benzodiazepines at 240 nm. For a more clear identification, diode-matrix detection is required [35, 36].

HPLC has shown his advantages due to the simple extraction procedure, as well as operation at appropriate temperature allow detection of non-volatile, polar and high mass molecules, which cannot be detected with other methodic like GS. Eluted drugs may be recovered for further tests because the detection system is not destructive [35-37].

1.8.2. High-performance liquid chromatography methods suitable for bromazepam, nitrazepam, and triazolam identification

Scientific literature has described various methods for the HPLC, which was to investigate the bromazepam, nitrazepam, and triazolam. They have widely used the drug in clinical trials assessing the pharmacokinetic properties of the materials and the industry in quality control. Table 8 shows methodologies that can be used for bromazepam, nitrazepam and triazolam for qualitative analysis in pharmaceutical preparations.

Table 8. HPLC methods suitable for bromazepam, nitrazepam, and triazolam qualitative analysis according to Clark’s analysis [32]

<table>
<thead>
<tr>
<th>Methodics</th>
<th>Bromazepam RT (min)</th>
<th>Nitrazepam RT (min)</th>
<th>Triazolam RT (min)</th>
</tr>
</thead>
</table>

chromatography is mainly stated on mechanisms of absorption, mass distribution, ion exchange, size exclusion or stereo-chemical interaction [6].
<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HI</td>
<td>-</td>
<td>2.96</td>
<td>4.38</td>
</tr>
<tr>
<td>HJ</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HK</td>
<td>2.99</td>
<td>1.49</td>
<td>1.83</td>
</tr>
<tr>
<td>HZ</td>
<td>-</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>HAA</td>
<td>-</td>
<td>16.9</td>
<td>17.4</td>
</tr>
<tr>
<td>HAX</td>
<td>-</td>
<td>6.3</td>
<td>6.4</td>
</tr>
<tr>
<td>HAY</td>
<td>-</td>
<td>6</td>
<td>6.7</td>
</tr>
</tbody>
</table>

**System HI**
- Column: ODS Hypersil (200x5mm i.d., 5 mm).
- Detector: DAD

**System HJ**
- Column: ODS Hypersil (200x5mm i.d., 5 mm).
- Detector: DAD
- Elution program: (70: 10: 20).

**System HK**
- Column: Silica Spherisorb (250x5mm i.d., 5 mm).
- Mobile phase: Methanol to which has been added 100 mL perchloric acid per liter.
- Detector: DAD

**System HZ**
- Column: C18 end-capped LiChrospher 100 RP-18e (125x4.0mm i.d., 5 mm), with precolumn LiChrocart 124-4.
Mobile phase: Add 146 mL triethylamine and about 750 mL phosphoric acid to 530 mL water. Adjust pH to 3.3 using a 10% potassium hydroxide solution and finally add 470 mL acetonitrile.

Flow rate: 0.6 mL/min.

Detection: DAD.

System HAA
- Column: C8 Symmetry (250x4.6mm i.d., 5 mm) with Symmetry C18 precolumn (20 mm).
- Column temperature: 30°C.
- Mobile phase: (A: B) phosphate buffer (pH 3.8)–acetonitrile.
- Elution programme: (85: 15) for 6.5 min to (65: 35) until 25 min to (20: 80) for 3 min, and back to initial conditions for equilibration for 7 min.

System HAX
- Column: Supelcosil LC-DP (250x4.6mm i.d., 5 mm).
- Eluent: (A: B: C) Acetonitrile–phosphoric acid (0.025% v/v) – triethylamine buffer.
- Isocratic elution: (25: 10: 5).
- Flow rate: 0.6 mL/min.
- Detection: DAD (λx229 nm).

System HAY
- Column: LiChrospher 100 RP-8 (250x4.0mm i.d., 5 mm).
- Eluent: (A: B: C) Acetonitrile–phosphoric acid (0.025% v/v) – triethylamine buffer.
- Isocratic elution: (60: 25: 15).
- Flow rate: 0.6 mL/min.
- Detection: DAD (λx229 nm).

According to UNODC “Recommended methods for the identification and analysis of barbiturates and benzodiazepines” [31]. The two suitable systems were:

**Table 9. HPLC methods suitable for bromazepam, nitrazepam and triazolam qualitative analysis.**
The review of the literature found studies that analyzed nitrazepam and triazolam, however, for bromazepam analysis, there were fewer data. Table 8 and 9 shows the HPLC methodology adapted to analyze all three pharmaceutical preparations separately, but the pharmaceutical’s retention times in many systems are very close to each other, so the mixture analysis described methods are not adequate. HPLC literature methodic was used as a base for the establishment and development of appropriate HPLC methodology suitable for bromazepam, nitrazepam and triazolam qualitative analysis in a mixture.
2. EXPERIMENTAL PART

2.1. Materials and methods

2.1.1. The object of investigation

In order to create an optimal qualitative analysis by TLC (thin layer chromatography) and HPLC (high-performance liquid chromatography) prepared medicinal mixtures of Bromazepam, Nitrazepam and Triazolam were studied. Materials have been extracted from “Bromazepam Lannacher” (G.L. Pharma GmbH), “Eunoctin” (Gedeon Richter Plc.), and “Halcion” (Pfizer H.C.P) (authorized in LT medicines) solutions.

2.1.2. Qualitative assessment using thin-layer chromatography (TLC) method

Equipment

Firstly, to identify the optimal solvent system was used the aluminum layer chromatographic plate (DS – Fertigfolien Alugram SIL G/ UV254) F60254 coated with silica gel, with a resin layer of 0,2 mm, size 10 x 20 cm. For further investigation, after identifying the most suitable solvent system has been used for more qualitative and accurate results - glass plates. The standard and tested solution have been applied with a loading system, which is a semi-automatic sampler (CAMAG Linomat 5), in where the sample is applied in a sequence forming dash-shaped stains. The sampling volume used was 10 μl. The chromatogram has been performed in a CAMAG Twin Chamber 20x20 chromatographic chamber. Solvent system volume of 100 mL.

Solvents

Analyzed solutions, as well as the standard solution, have been prepared using methanol solvent. For the preparation of mobile solvent system, such solvents were used as cyclohexane, methanol, diethylamine, toluene, sulfuric acid, acetonitrile.

Visualizer

Dried chromatographic plates are observed with:
- UV light (254 nm). Further obtained spots of the mixture prepared from medical substances are identified for Rf values using the imaging device CAMAG TLC Visualizer connected with software VideoSCan equipment.

**Standard solutions**

Standard solutions of the tested substances have been prepared all by dissolution of the standard in methanol. Has been received 3 standard solutions of a concentration of 0, 1 mg/ml: reference solution bromazepam (Sigma–Aldrich, JAV), nitrazepam (Sigma–Aldrich, JAV), triazolam (Sigma–Aldrich, JAV). Reference solutions: bromazepam RSB, nitrazepam RSN, triazolam RST.

A reference mixture was prepared consequently (RS MBNT 1) by using 1 ml of each standard solution prepared.

**Test solutions**

The solution for analysis has been prepared from the drugs obtained in the pharmacy stores in Lithuania. Bromazepam solution (B1) was produced from the medicinal product “Bromazepam Lannacher” (G.L. Pharma GmbH), which are 3 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to a volumetric flask and dissolute up to 30 ml with methanol. The suspension has been further with help of an ultrasound bath mixed well for 10 min. The precipitate obtained has been separated by centrifuge. The 30 ml solution has been further filtrated with PVDF filter with pore size of 0, 45 mm. The resulting solution has been transferred to cork closed flask and used for upcoming researches.

Nitrazepam standard solution (N1) has been prepared from the medicinal product “Eunoctin” (Gedeon Richter Plc,), which are 10 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to a volumetric flask and dissolute up to 100 ml with methanol. The suspension has been further with help of an ultrasound bath mixed well for 10 min. The precipitate obtained has been separated by centrifuge. The 100 ml solution has been further filtrated with PVDF filter with pore size of 0, 45 mm.
mm. The resulting solution has been transferred to cork closed flask and used for subsequent researches.

Triazolam standard solution (T1) has been prepared from the medicinal product “Halcion 250 μg” (Pfizer H C.P), which is 250 μg dosage tablets. 2 tablets have been crushed and grinded well in the mortar. The powder obtained has been transferred to a volumetric flask and dissolve up to 5 ml with methanol. The suspension has been further with help of an ultrasound bath mixed well for 10 min. The precipitate obtained has been separated by centrifuge. The 5 ml solution has been further filtrated with PVDF filter with pore size of 0.45 mm. The resulting methanol solution has been transferred to cork closed flask and used for further research.

Analysis

Reference solutions – RSB 1, RSN 1, RST 1 and their mix RS MBNT 1 were used to further identify the most suitable and optimal conditions for separation. The chromatographic plates coated with silica gel using the semi-automatic sampler CAMAG Linomat 5 have spread the samples. The samples are introduced on the plates on the starting line (1.5 cm from the bottom of the plates). The plates with introduced samples are transferred into the prepared chromatographic chamber with the assigned solvent system (SS) and are performed up until the SS rises up to 10 cm from the bottom line. (The start point and the end point should be measured prior to performance analysis). The chromatographic plates are removed, dried and visualized with UV light (254 nm). The identified and separated substances have been detected and its retention index (Rf) values are calculated. The measurement of TLC – (Rf) retention factor of the tested substance is expressed in the formula by (L1) - the displacement distance from the starting point of the solvent system to (L2) - the displacement distance of the substance from the bottom line and obtained as a ratio of those two values.

$$Rf = \frac{L1(mm)}{L2(mm)}$$

The experimental analysis is to determine the most appropriate solvent system for separation of bromazepam, nitrazepam and triazolam mixtures. Therefore, in order to find the most appropriate methodology it has been carried multiple studies with separate solutions, the
reference solutions (RSB 1, RSN 1, RST 1), the tested solutions (B1, N1, T1) and the subsequent mixture solution (RSMBNT 1, MBNT) and were applied for accurate results on the chromatographic plates during investigation and analysis. On the other hand, the chromatographic conditions have been kept constant (same quantitative compositions, application methodic, solvent volumes, chamber type). Chromatography time has to reach 10 cm from the starting line; plates further were dried and visualized under UV light (254 nm). The stains were identified and their Rf calculated and compared with the standard solutions.

2.1.3. Qualitative and quantitative assessment using high-performance liquid chromatography method

Equipment

Qualitative evaluation has been performed with help of high-performance liquid chromatography (HPLC); ideal conditions have been acquired using chromatograph (Waters 2695) with a photodiode array detector (Waters 996, at wavelength 200-400nm range). Mixtures of solution separation have been performed with chromatographic column ACE C18 (2, 1 mm x 5, 0 cm) with sorbent particle size of 5μm.

Solvents

For all necessary preparations of standard solutions have been used methanol. For chromatography mobile phase has been used: acetonitrile, water purification system, sulfuric acid buffer 0, 1% aqueous solution.

Standard solutions

Standard solutions of the tested substances have been prepared all by dissolution of the standard in methanol. Has been received 3 standard solutions of a concentration of 0, 1 mg/ml. Reference solutions: bromazepam RSB, nitrazepam RSN, triazolam RST.

A reference mixture was prepared consequently (RS MBNT 2) by using 1 ml of each standard solution prepared.

Test solutions
The solution for analysis has been prepared from the drugs obtained in the pharmacy stores in Lithuania. Bromazepam solution (B2) was produced from the medicinal product “Bromazepam Lannacher” (G.L. Pharma GmbH), which are 3 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to a volumetric flask and dissolute up to 10 ml with methanol. The suspension has been further with help of an ultrasound bath mixed well for 10 min. The precipitate obtained has been separated by centrifuge. The 10 ml solution has been further filtrated with PVDF filter with pore size of 0, 45 mm. The resulting solution has been transferred to cork closed flask and used for upcoming researches.

Nitrazepam standard solution (N2) has been prepared from the medicinal product “Eunoctin” (Gedeon Richter Plc.), which are 10 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to a volumetric flask and dissolute up to 20 ml with methanol. The suspension has been further with help of an ultrasound bath mixed well for 10 min. The precipitate obtained has been separated by centrifuge. The 10 ml solution has been further filtrated with PVDF filter with pore size of 0, 45 mm. The resulting solution has been transferred to cork closed flask and used for subsequent researches.

Triazolam standard solution (T2) has been prepared from the medicinal product “Halcion 250 μg” (Pfizer H.C.P.), which is 250 μg dosage tablets. 2 tablets have been crushed and grinded well in the mortar. The powder obtained has been transferred to a volumetric flask and dissolute up to 5 ml with methanol. The suspension has been further with help of an ultrasound bath mixed well for 10 min. The precipitate obtained has been separated by centrifuge. The 10 ml solution has been further filtrated with PVDF filter with pore size of 0, 45 mm. The resulting methanolic solution has been transferred to cork closed flask and used for further research.

Analysis

For analysis, a mixture of solutions has been prepared from bromazepam, nitrazepam, and triazolam by taking 2, 2 ml and preparing 6 samples, which were consequently diluted each time by 2, 2 ml of methanol and an initial mix solution from all three tested preparations with
concentration of 0.1 mg/ml and therefore has been analyzed with high performance liquid chromatography under optimized conditions. Bromazepam, nitrazepam, and triazolam mixture RS MBNT2 made from standard solutions for analysis with HPLC methodic required optimizing the composition of the eluent and the elution method (isocratic or gradient mode). However, the injection mode remained constant 10 μl. Bromazepam, nitrazepam, triazolam mixtures have been identified by UV absorption spectrum, light absorption at a range from 200-600nm wavelength.

When the most appropriate conditions have been set and adapted, pharmaceutical solutions have been analyzed in order to check the suitability of the methodology chosen for separation of bromazepam, nitrazepam, and triazolam medicinal product.
3. RESULTS AND DISCUSSION

3.1. Thin-layer chromatography (TLC) methodic selection

Eluent selection

A sequence of experimental analysis has been performed in order to determine the most appropriate solvent system (SS) which will clearly show the separation and identification of bromazepam, nitrazepam and triazolam and their mixtures subsequently. In order to find the most suitable, several solvents have been used, and their ratio composition has been adjusted to, therefore, find the most appropriate solvent system. According to the reference solution, it has been identified the right and approved systems. And has been selected 3 most fitting SS system suitable for appearing separation of the medicines (Table 10).

Table 10. Average bromazepam, nitrazepam and triazolam Rf in different solvent systems.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Bromazepam av. Rf</th>
<th>Nitrazepam av. Rf</th>
<th>Triazolam av. Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexane - methanol- diethylamine (75:15:10) (SS – 1)</td>
<td>0,33</td>
<td>0,27</td>
<td>0,29</td>
</tr>
<tr>
<td>Chloroform – acetone (80:20) (SS - 2)</td>
<td>0,03</td>
<td>0,01</td>
<td>0,01</td>
</tr>
<tr>
<td>Chloroform- methanol (90:10) (SS – 3)</td>
<td>0,41</td>
<td>0,42</td>
<td>0,4</td>
</tr>
</tbody>
</table>

Statistic evaluation

For investigation and assessment of the reliable methodic and system to choose, analysis of mix solutions (RS MBNT) was performed with repeating 3 times each. The obtained data was analyzed statistically by calculating the Rf values averages obtained from 3 repeated analysis solutions, standard deviation, a relative error at a confidence level of 0,95, as well as a confidence interval when the error p=0,05.
As we can see from the diagram above (Diagram 1), systems: SS-2 cannot be used for analysis of medicinal preparations, because of absence in the difference in Rf values of nitrazepam and triazolam. SS-3 system is not appropriate for analysis, because of its very low Rf resulting value while performing analysis (average Rf value <0.01).

It has been found that among the most appropriate solvent system for bromazepam, nitrazepam and triazolam mixture separation SS-1 was the most suitable. Using SS-1 mobile phases has separated mixture components with a significantly visible difference in Rf, comparing to other methodic. Average Rf value difference between (bromazepam and nitrazepam) was >0.06, (bromazepam and triazolam) >0.04, and (nitrazepam and triazolam) >0.02.
Fig. 7. SS – 1 under UV light (254 nm) analysis performed on reference bromazepam (RS B1), nitrazepam (RS N1), triazolam (RS T1) and their mix (RS BNT1).

3.2. Medicines analysis using TLC method

When the optimal conditions have been set for the solvent systems and the visualization methods have been selected, the analysis has been performed on the medicinal preparations obtained from Lithuanian pharmacies. Analysis has been performed on the SS-1 and visualized with UV light (254 nm).

Table 11. Analyzed and reference solutions Rf values in the optimal solvent system for separation.

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Bromazepam av. Rf</th>
<th>Nitrazepam av. Rf</th>
<th>Triazolam av. Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexane - methanol- diethylamine (75:15:10) (SS – 1)</td>
<td>0,33</td>
<td>0,34</td>
<td>0,27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,27</td>
<td>0,29</td>
</tr>
</tbody>
</table>
Fig. 8. System SS-1 at UV 254nm, Separation of analyzed medicinal preparations bromazepam – B1, nitrazepam – N1 and triazolam – T1.

Analyzed preparations extracted from pharmaceuticals shows that the Rf values obtained during analysis fit into the confidence interval set to the reference solutions, therefore meets the requirements for solvent system SS-1, bromazepam obtained Rf = 0.33, nitrazepam obtained Rf = 0.27 and triazolam obtained Rf = 0.29 thus it can be concluded that SS-1 is valid for qualitative analysis of bromazepam, nitrazepam, and triazolam.

3.3. High-performance liquid chromatography (HPLC) methodic selection

Detector selection

For the most accurate assessment of our selected tested drug mixtures, a method of high liquid chromatography is used. A detector DAD - Diode Array Detection (SPD-M20A). With the help of detector can be determined the absorption peaks of analytes with UV light (200-600 nm) in the appropriate UV light spectrum. Therefore a qualitative evaluation of the mixtures can be performed. Accounting this criterion, as well as an appropriate retention time chosen we can reach a high accurate qualitative assessment of mixtures tested.
Column selection

In order to determine the most appropriate chromatography column, investigated pharmaceutical mixture (RSBNT 2) were observed using column: Sunfire C18 (length 15 cm, internal diameter 3.0 mm, sorbent particle size 3.5μm) Supelco LC18 (length 15 cm, inner diameter 4.6 mm; resin particle size of 5.0μm) and an ACE C18 (length 25 cm, inner diameter 4.6 mm, the resin particle size 5μm). Isolation best achieved through the ACE C18 chromatography (25 cm × 4 mm × 5μm) column chromatography.

Eluent system selection

In order to separate and identify the drug substances components of the medicinal solution, we have analyzed mixture solution (RSBNT2) we have performed chromatography tests using different eluents systems. Experiments did not reach the maximum isolation, using the literature described conditions; therefore it was necessary to modify the systems in order to get optimal results. The solvent system was evaluated by the retention time of the analytes, the symmetry of the peaks and baseline stability.

Bromazepam, nitrazepam and triazolam mixtures, the separation was achieved by eluting with solvent systems consisting of 0.1 % sulphuric acid aqueous solution (A) and acetonitrile (B) by changing the quantitative composition gradient (proportions described in Table 12).

Table 12. Eluent quantitative composition gradient variation over time.

<table>
<thead>
<tr>
<th>Chromatography time (min)</th>
<th>Eluent speed (ml/min)</th>
<th>Sulfuric acid buffer 0.1% (A)</th>
<th>ACN – Acetonitrile (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>1.0</td>
<td>98.0</td>
<td>2.0</td>
</tr>
<tr>
<td>1.00</td>
<td>1.0</td>
<td>98.0</td>
<td>2.0</td>
</tr>
<tr>
<td>20.00</td>
<td>1.0</td>
<td>2.0</td>
<td>98.0</td>
</tr>
</tbody>
</table>
Examination of separation of mixtures was performed when the mobile phase flow rate was 0, 1 ml/min, 0, 2 ml/min, and 0, 4 ml/min, the optimal separation was observed when the flow rate of the eluent was 1, and 0 ml/min. General chromatographic analysis duration was 29 minutes.

### 3.3.1. Adapted HPLC methodic validation

**Specificity**

Method of specificity is methodic of distinguishing the tested substances from impurities and other material, which can be present in the prepared drug composition. Tested mixtures are improved separating with HPLC methodic with photodiode array detector. Analyte specificity is demonstrated by comparing the standard and analyte retention time and spectral overlaps.
Fig. 9. Standard solution chromatogram RS B2 – Bromazepam (retention time 10.905), RS N2 – Nitrazepam (12.829), RS T2 – Triazolam (14.537).

Fig. 10. Standard solution chromatogram RS of B2 - Bromazepam (retention time 10.905).

Fig. 11. Standard solution chromatogram RS of N2 - Nitrazepam (retention time 12.829).
Fig. 12. Standard solution chromatogram RS of T2 - Triazolam (retention time 14.537).

Method precision

In order to evaluate the precision of the method during analysis of mixtures of the results obtained we considered two major factors: repeatability and reproducibility. Repeatability describes the accuracy of the results of the analysis which was performed, therefore the tests were performed a couple of times. For this investigation, the standard mixtures were performed four times. Reproducibility describes the accuracy of the results, which is performed under the same conditions, but on different days. HPLC is a method used for evaluation of reproducibility in which 8 tests were performed on two different days.

Linearity

Linearity – the results depend upon the concentration of the mixtures tested. The calibration curve should be drawn from at least 5 points present, and then the calibration curve can be performed. Bromazepam calibration curve (fig.13) is composed out of 7 points, respectively nitrazepam (fig. 14) and triazolam (fig.15) as well. The resulting characteristics are shown in the (table 13). Obtained results are a showing a very strong linear correlation, which can determine that it is a suitable quantitative determination of mixtures. In analyzed mixtures, we observed that the correlation coefficient is higher than 0, 99.
Table 13. Analytes characteristic of the calibration curves

<table>
<thead>
<tr>
<th>Analyzed medicine</th>
<th>Correlation coefficient</th>
<th>Calibration curve equation</th>
<th>Linearity limit (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromazepam</td>
<td>0.9999908</td>
<td>$f(x)=45074.9x+8125.77$</td>
<td>0.9999734 - 0.9999916</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>0.9999867</td>
<td>$f(x)=35455.8x+3721.61$</td>
<td>0.9999734 - 0.9999916</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0.9999916</td>
<td>$f(x)=68588.5x-724.393$</td>
<td>0.9999734 - 0.9999916</td>
</tr>
</tbody>
</table>

Fig. 13. Bromazepam calibration curve, calibration curve equation: $f(x) = 45074.9x + 8125.77$

Fig. 14. Nitrazepam calibration curve, calibration curve equation: $f(x) = 35455.8x + 3721.61$
Fig. 15. Triazolam calibration curve, calibration curve equation: $f(x) = 68588.5x - 724.393$

**LOD and LOQ limits**

LOD (limit of detection) is considered as the calculation of the smallest possible amount of analyte that can be observed but not necessarily quantified as an exact value. It is usually calculated based on the known concentration of the analyte to be analyzed and the minimum level at which the analyte is detected is then set.

LOQ (limit of quantification) is the smallest amount of analyte that can be reliably detected in the sample with accuracy and precision. The limit of quantification is a parameter of quantitative analyzes for low levels of compounds in sample matrices and is used, in particular, for the determination of impurities and/or degradation products.

**Table 14. Alprazolam, Estazolam and triazolam limits of detection LOD and LOQ values.**

<table>
<thead>
<tr>
<th>Analyzed solution</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromazepam</td>
<td>0.078</td>
<td>0.026</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>0,017</td>
<td>0,056</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0,010</td>
<td>0,020</td>
</tr>
</tbody>
</table>

3.4. Medicines analysis using HPLC method

3.4.1. Qualitative medicines analysis

Once the optimum conditions were set at which can be identified bromazepam, nitrazepam, and triazolam in the mixture, the methodology was applied in analyzing solutions B2, N2 and T2 made of pharmaceutics medicines from pharmacies located in Lithuania.

Examination of medicinal product “Bromazepam Lannacher” solution, bromazepam in the chromatogram were identified by the following criteria: a retention time (10.903), which is close to the reference solution for a retention time of bromazepam; UV light absorption spectrum that is identical to the literature bromazepam UV absorption spectrum (Fig 16).

![Graph](image)

**Fig.16. Analyzed medicinal solution B2 (Bromazepam)**

Analyzing medicinal product “Eunoctin”, nitrazepam was identified on the chromatogram with a retention time 12.823 min and a UV light absorption spectrum that is identical to the literature UV abortion spectrum. (fig.17).
Analyzing medicinal product “Halcion”, triazolam was identified on the chromatogram with a retention time 14.529 min and a UV light absorption spectrum that is identical to the literature triazolam UV absorption spectrum (fig.18).
3.4.2 Quantitative medicines analysis

In order to assess the suitability of selected HPLC method for the quantitative determination of test materials were prepared calibration curve of light absorption peak height dependence on substance concentration. The higher the tonnage, the higher peak. The produced standard solutions were prepared at different concentrations to create calibration curve solutions by diluting standards in half. Bromazepam (Fig.13), nitrazepam (Fig.14), and triazolam (Fig.15) calibration curve made from 7-point concentration range 0.9999734 - 0.9999916 mg/ml.
CONCLUSION

1. In an analysis of literature, no suitable methodic was found for the separation of mixture solutions containing medicinal product: bromazepam, nitrazepam, and triazolam for thin layer chromatography. But through experimental work conduction it has been found that the most suitable system for analysis of mixture in order to separate its components SS-1 was the most appropriate one (Cyclohexane: methanol: diethylamine (75:15:10). After re-evaluation of the reference solution, it has been stated to obtain medicinal solutions Rf and had stated average Rf values: bromazepam (RF=0, 33), nitrazepam (RF=0, 29) and triazolam (RF=0, 27). Upon repetition of analysis, it has been stated that the Rf value hasn’t exceeded repetition error p <0, 5 limitation. Chosen TLC methodic can be concluded to be acceptable for qualitative analysis of bromazepam, nitrazepam, and triazolam.

2. In an analysis of literature, no suitable methodic was found for the separation of mixture solutions containing medicinal product: bromazepam, nitrazepam, triazolam for high-performance liquid chromatography for quality assessment. But through experimental work, bromazepam, nitrazepam, triazolam has been separated with HPLC using ACE C18 (2, 1 mm x 5, 0 cm, 5μm) column, has been adjusted eluent gradient (Sulfuric acid buffer 0, 1% and ACN). Eluent speed 0, 1 ml/min and an injection volume of 10 μl. Medicines have been identified with help of diode array detector. After performing analysis with a reference solution, analysis with medicinal mixture has been examined and has stated the retention time: bromazepam (10.903 min), nitrazepam (12.823 min) and triazolam (14.529 min). Retention time upon repetition of analysis has not exceeded the relative error of p <0, 05 limitation.

3. When the optimum conditions for analysis by thin layer chromatography have been adjusted, the selected methodic has been applicable for the medicinal preparation of “Bromazepam Lannacher” (G.L. Pharma GmbH), “Eunoctin” (Gedeon Richter Plc.), “Halcion” (Pfizer H.C.P) for qualitative assessment. In analysis was concluded that medicinal solutions, extracted from the medicines product had a correspondent average Rf values which corresponded to the confidence intervals of the most suitable system SS-1. Therefore, it was concluded that thin layer chromatography method was suitable to separate bromazepam, nitrazepam, triazolam in pharmaceutical preparations.
4. The chosen high-performance liquid chromatography methodology was proven to be suitable for the medicinal preparation of “Bromazepam Lannacher” (G.L. Pharma GmbH), “Eunoctin” (Gedeon Richter Plc.), and “Halcion” (Pfizer H.C.P) qualitative assessment. Bromazepam, nitrazepam and triazolam medicinal products have been established in accordance with:

- Reference solution and analyzed solution retention time
- Reference solution and analyzed solution UV light absorption spectrum. UV absorption spectrum of analyzed solutions has fit with UV Spectrum of the reference solution. The chosen method is suitable for separation of bromazepam, nitrazepam and triazolam qualitative assessment excreted from medicinal preparations.

5. Applied high-performance liquid chromatography has been used to perform calibration curve from the resulting bromazepam, nitrazepam, and triazolam qualitative evaluation, and therefore have shown that the limits of detection of bromazepam are 0,078 µg/ml, nitrazepam 0,017 µg/ml, triazolam 0, 010 µg/ml. Limit of quantification of bromazepam is 0, 026 µg/ml, nitrazepam 0, 056 µg/ml, triazolam 0, 020 µg/ml.
PRACTICAL RECOMMENDATIONS

Taking into consideration the results obtained in thin-layer chromatography and high-performance liquid chromatography, it can be concluded that they are suitable for qualitative evaluation of bromazepam, nitrazepam and triazolam preparations with drugs alone or in solution mixtures. It is advisable to develop extensive studies to adapt the methodology chosen by thin layer chromatography, quantitative analysis. In order to expand the use of selected methods of toxicological analysis in practice, it is advisable to conduct detailed studies to evaluate the effectiveness of methods for determining the materials in question in pharmaceutical preparations.
REFERENCES


