YELYZAVETA KRAVCHUK

ANALYSIS OF PSYCHOTROPIC MEDICATIONS TRIAZOLAM, ESTAZOLAM AND ALPRAZOLAM MIXTURE USING THIN-LAYER CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHODS

Master's thesis

Kaunas, 2017
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Master's thesis

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Date (________________)

Kaunas, 2017
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SUMMARY

Master Thesis of Y. Kravchuk “Analysis of psychotropic medicines triazolam, estazolam, and alprazolam mixture using thin – layer chromatography and high – performance liquid chromatography “. Scientific supervisor PhD. Andrejus Ževžikovas; Lithuanian University of Health Sciences, Faculty of Pharmacy, Department of Analytical and Toxicological chemistry. – Kaunas

Aim of the thesis: To optimize thin-layer chromatography and high-performance liquid chromatography methods for triazolam, estazolam, alprazolam and their mixture qualitative analysis.

Objectives and methods: For optimization of TLC method for triazolam, estazolam, alprazolam and their mixture solutions in methanol were analyzed. For mobile phase were used: toluene, cyclohexane, methanol, diethylamine, triethylamine, ethyl acetate, chloroform (solvents). For spots visualization were used: UV light lamp (254 nm; 365nm) or Dragendorff reagent (modified by Munje). Optimized methods were tried with pharmaceutical products “Xanax” (Pfizer H.C.P), “Estazolam TC” (Polfa Tarchomin), “Halcion” (Pfizer H.C.P) (authorized in LT medicines) solutions.

For optimization HPLC method triazolam, estazolam, alprazolam and their mixture solutions 0,1mg/ml in methanol were analyzed. Chromatograph Waters 2695 with photo diode array detector Waters 996 (200-600 nm wave length) were used for qualitative determination. Analysis was performed by using Sulfuric acid buffer 0,1 % and Acetonitrile solution. Optimized method was applied in analysis of pharmaceutical products “Xanax” Pfizer H.C.P, “Estazolam TC” (Polfa Tarchomin), “Halcion” (Pfizer H.C.P) (authorized in LT medicines) solutions.

Results: The best mobile phases for triazolam, estazolam, alprazolam mixture qualitative analysis using TLC is system “Cyclohexane-methanol-diethylamine” (75:15:10) solvents system with appropriate ratio). This mobile phases is suitable for examined substances separation qualitative analysis in mixture and determination in pharmaceutical products.

HPLC method was developed using ACE C18 (25 cm × 4,6 mm × 5 µm) chromatographic column. Gradient elution mode was used (mixture of 0,1% sulfuric acid buffer solvent and ACN solvent). Flow rate 1 ml/min. Injection volume 10 µl. Photo diode array detector (200-600 nm wave length). Triazolam, estazolam, alprazolam absorption of UV light was similar as in scientific literature. HPLC method is suitable for examined substances qualitative analysis in mixture and determination in pharmaceutical products.

Key words: triazolam, estazolam, alprazolam, thin-layer chromatography, high-performance liquid chromatography, qualitative determination.
ABBREVIATIONS

HPLC – high – performance liquid chromatography

Rf – retention index

RS – reference standard

RT – retention time

SS – solvent system

TLC – thin – layer chromatography

UV – ultraviolet light
AIM AND WORK TASKS

The aim: to optimize TLC and HPLC methodics, suitable for triazolam, estazolam and alprazolam qualitative evaluation.

The main goals of master’s thesis work:

1. To optimize the TLC methodic which will be suitable for triazolam, estazolam and alprazolam and their mixture qualitative evaluation and mixture separation.
2. To optimize the HPLC methodic which will be suitable for triazolam, estazolam and alprazolam and their mixture qualitative evaluation and mixture separation.
3. To adapt the optimized TLC and HPLC methodics for triazolam, estazolam and alprazolam excreted from medicines for qualitative evaluation.
4. Validation of suitable HPLC methodic for its usage in quantitative analysis of investigated pharmaceuticals.
1. LITERATURE OVERVIEW

1.1. Triazolam, estazolam and alprazolam prevalence of use

1.1.1. Triazolam, estazolam and alprazolam prevalence of use in Lithuania and Baltic countries.

According to the Baltic Statistic Medicines report in the Baltic countries (Estonia, Latvia and Lithuania) the last four-year consumption 2013, 2014, 2015, 2016 the general usage of antipsychotic drugs had a tendency of augmentation by DDD/1000/day. Lithuania has the highest consumption among the three countries.

![Consumption of antipsychotics (N05A)](image)

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

Anxiolytics consumption was 41,37 DDD/1000/day in 2013, has increased to 41,60 DDD/1000/day in 2014, 20,960 DDD/1000/day in 2015 and consequently decreased to 40,733 DDD/1000/day in 2016 in Lithuania, however the usage among the Baltic countries, clearly shows that from 2013 up to 2016 there is strong correlation in usage of alprazolam among the three countries, although Lithuania remains stably the country with significant increase and the most usage of alprazolam [1,2].

**Table 1. Consumption of Alprazolam in 2012, 2013, 2015 per DDD/1000/day in Baltic countries.**

<table>
<thead>
<tr>
<th>Alprazolam</th>
<th>2013 (DDD/1000/day)</th>
<th>2014 (DDD/1000/day)</th>
<th>2015 (DDD/1000/day)</th>
</tr>
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<tr>
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</tbody>
</table>
It has been reported that usage of alprazolam in 2016 reached up to 8,851 DDD/1000/day.

![Consumption of anxiolytics (N05B)]

**Fig. 2 Consumption of anxiolytics in Baltic countries in year 2013, 2014, 2015 per DDD/1000/day.**

Usage of Hypnotic and sedatives benzodiazepine derivate in Lithuania accounted for 2,018 DDD/1000/day in 2013, 2,275 DDD/1000/day in 2014, 2,702 DDD/1000/day in 2015 consequently reaching up to 2,979 DDD/1000/day.

**Table 2. Consumption of Estazolam in 2012,2013,2015 per DDD/1000/day in Baltic countries.**

<table>
<thead>
<tr>
<th></th>
<th>2013 DDD/1000/day</th>
<th>2014 DDD/1000/day</th>
<th>2015 DDD/1000/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estonia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Latvia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lithuania</td>
<td>0,064</td>
<td>0,055</td>
<td>0,048</td>
</tr>
</tbody>
</table>

**Table 3. Consumption of Triazolam in 2012,2013,2015 per DDD/1000/day in Baltic countries.**

<table>
<thead>
<tr>
<th></th>
<th>2013 DDD/1000/day</th>
<th>2014 DDD/1000/day</th>
<th>2015 DDD/1000/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estonia</td>
<td>0,05</td>
<td>0,06</td>
<td>0,08</td>
</tr>
<tr>
<td>Latvia</td>
<td>0,15</td>
<td>0,21</td>
<td>0,25</td>
</tr>
</tbody>
</table>
It has been reported that usage of estazolam in 2016 has decreased to 0.046 DDD/1000/day since 2013, which has been shown to be the highest amount used of estazolam for the last couple of years, on the other hand triazolam in 2016 shown increase reaching up to 2.235 DDD/100/day, which is two times higher than in 2013. According to data, Lithuania remains the leading country in usage of triazolam and estazolam, however Estonia shows the greatest report in usage of hypnotics and sedatives [1,2].

| Lithuania | 1.14 | 1.49 | 1.91 |

**Fig. 3 Consumption of hypnotics and sedatives in Baltic countries in year 2013, 2014, 2015 per DDD/1000/day.**

### 1.1.2 Triazolam, estazolam and alprazolam prevalence of use in other countries

In 2006 the International Narcotic Control board has noted that Europe has been seen to be the most consummative in benzodiazepines, which include 37 S-DDD in 2001–03 anxiolytics which rose to around 46 S-DDD in 2005–06, and then dropped to around 42 S-DDD in 2007–09. In 2007, global consumption of anxiolytics was around 22 billion S-DDD, which first rose to around 25 billion S-DDD and then dropped to around 21 billion S-DDD in 2009 [4]. For sedatives it was approximately 24 S-DDD in 2005–07 up which fell to 22 S-DDD in 2006–08 compared with other part of the world, however Australia has reached upon the same consumption due to extensive use of alprazolam.

Statistic show according to the National Survey on drug use and health in 2012 that alprazolam prevalence of use accounted for up to 10% of people with age from 18-25, which has been a double of 5.7% people aged 26 and above [5].
The pharmacy corporation in Sweden in 2012 notes that about 50 DDD/1000/day of benzodiazepines has been consumed for population, according to which accounts 28% of anxiolytics (including alprazolam and others) and 12% hypnotics (triazolam, nitrazepam). Overall the rates of benzodiazepines prescription had varied in contrast to Sweden from 43 to 62 DDD/1000/day [6,7,10].

1.1.3 Triazolam, estazolam and alprazolam comparison of prevalence of use in separate countries

Alprazolam is available in many countries and all regions. In 2011, more than 100 countries reported import or use of the substance. Global calculated consumption, which had averaged 4.8 billion S-DDD during the years of 2000-2005, reached an average of 8.5 billion S-DDD during the period 2009-2011. The countries reporting the largest consumption of alprazolam in absolute terms in 2012 were the United States (2.2 billion S-DDD), Hungary (184 million S-DDD) and Portugal (119 million S-DDD), triazolam and estazolam are harder to identify, as alprazolam accounts to be the most frequently drug consumed on the market among different countries, but it has been found that in 2011, alprazolam stated for 36 per cent (6.6 billion S-DDD) globally of all the total anxiolytics present on market, triazolam (987 million S-DDD) 14 per cent, estazolam (473 million S-DDD) 7 per cent from total hypnotics present [9].

Findings by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) also show that benzodiazepine use increased in the past decade, from 37 daily doses per 1,000 citizens in 2001-2003 to 42 daily doses in 2007-2009 [10].

1.2. Statistics of poisoning using triazolam, estazolam and alprazolam

Poisoning of benzodiazepines, particularly triazolam, estazolam and alprazolam usually is caused by consumption of the drug in bigger doses than prescribed, however it is very unusual to cause death, unless the drug is used simultaneously with other drugs, those include alcohol, barbiturates, opioids or tricyclic antidepressant which may lead to severe health consequences such as coma or death. Common it is very rare to result in death after hospital admission [11,12]. Admissions which are cause by poisoning are usually treated by supportive care, most frequent symptoms include ataxia, impaired balance, not clear speech, on the other hand it can be present more severe symptoms such as respiratory depression [12].
As benzodiazepines is one of the class, which is commonly prescribed, it has shown very high percentage of self-poisoning due to inappropriate drug high dosage [13,14]. Due to different levels of sedation, benzodiazepines produce different toxicity. An Australian study (2004) had results based on admissions between 1987 and 2010, has concluded that alprazolam was the most common drug in overdose between other benzodiazepines, with an induced toxic effect. [15] An Annual Report of the American Association of poison control has cited that about 34 deaths has resulted in alprazolam between 1992-2001, which is a lot higher compared with other benzodiazepines prescribed such as diazepam [16].

British Journal of clinical pharmacology in 2004 has brought statistical analysis that alprazolam has been a lot more dangerous and toxic than other benzodiazepines in overdose, and had the higher death increase in the 2003-2009 consequent years [17,20]. A study in 2012 from the American Journal of Addiction in west Virginia in 2005-2007 indicated that those deaths reported from alprazolam overdose shows that more than 57,5 % didn’t have a documented prescription. It has been stated that majority of cases has been accidental, however increased dosage was also a mean to commit suicide in those people who were diagnosed priory with depression or anxiety [14].

In USA, alprazolam is the most frequently prescribed benzodiazepine [19]. A governmental study SAMHSA, has concluded that 35% of alprazolam drug related visits to emergency has been due to benzodiazepines misuse or overdose leading to poisoning cases, the rate of poisoning has increased for 36% in between 2004 and 2006. Substance and mental health administration states that 50% of hospitalization were caused due to simultaneous usage of benzodiazepines with alcohol and other drugs in 2011 [18].

1.3. Triazolam, estazolam and alprazolam toxicity

The drug class of benzodiazepines, to which belong alprazolam, triazolam and estazolam may have variable potency, duration effect, presence or absence of active metabolite and as well as their clinical use. As for the drugs chosen in this work, alprazolam has a half-life – 6,3 to 26,9 (h), with no active metabolite and an oral dose that range between 0,25- 0,5 mg, estazolam half-life – 8 to 28 (h), with no active metabolite and an oral dose that range between 1- 2 mg, triazolam half-life – 1,5 to 5,5 (h), with no active metabolite and an oral dose that range between 0,125- 0,5 mg. Those benzodiazepines are very highly bound in protein (80-100%).
Alprazolam is a benzodiazepine which has a toxic effect on the central nervous system depression. Comparable low doses can cause dizziness, lack of coordination and visual disturbances, muscle weakness. Alprazolam high dose (0.10 mg / l) especially in combination with alcohol or other central nervous system-active (opioid analgesics, tricyclic antidepressants, cocaine, methamphetamine, etc.), and may cause hallucinations, ataxia, loss of consciousness, coma, respiratory centre depression, hypotension, reflex tachycardia and death [21, 22 23]. Even up to 94.9% of fatal poisoning alprazolam cases patients are identified and others not only with alprazolam. The most common analgesics (64.6%), other benzodiazepines (44.4%) and alcohol (34.5%) [21].

Compared with other benzodiazepines, alprazolam poisoning cases, patients experience greater toxicity. Poisoning alprazolam, 2.06 times more patients were in need of urgent intensive care than other benzodiazepines poisoning. The average duration of the intensive monitoring of the patient after poisoning alprazolam was 19 hours - it is 1.27 times higher compared to other benzodiazepines caused by poisoning. Flumazenil treatment and artificial lung ventilation were patient’s poisoned with alprazolam, it was covered by 8% and 11% higher than other cases of poisoning. Twelve percent of patients had coma (GCS <9) as compared with 10% for other benzodiazepine overdose [24].

1.3.1. Mechanism of toxicity

Usually benzodiazepines toxicity is produced as a result of enhanced action of the inhibitory neurotransmitter of GABA, therefor such events like sedation, depression of spinal reflex and anticonvulsant effects occurs, and consequently may lead to severe respiratory arrest and coma. Symptoms of such toxicity may include: drowsiness, nystagmus, confusion, ataxia, amnesia, hypotension, respiratory depression and other [28,29].

1.3.2. Toxic dosage

Toxic therapeutic ratio of benzodiazepines is considered to be high, as an example triazolam result in respiratory arrest has been reported after ingestion of 5 mg. Alprazolam and triazolam which are newer short acting benzodiazepines tend to cause respiratory arrest more likely and more often rather than others benzodiazepines [25]. The cases are very rare of having acute toxicity which is resulting in death, on the other hand the risk are augmenting of undesirable toxic effect in presence of benzodiazepines with other CNS depressant, which include ethanol, barbiturates and others [25,26]. In
those cases the additive effect of benzodiazepines and CNS depressant especially if used in elderly will lead to aggravation and respiratory failure. Generally, up to 30-40% of cases with poisoning of benzodiazepines consider with combination of benzodiazepines with other drugs. Some other effect, such as hypotensive effect of benzodiazepines may rise adverse cardiovascular effects of cardio depressants.

It has been reported that some drug interactions with benzodiazepines maybe cause or lead to toxicity events, those include as an example isoniazid, which lead to inhibition of elimination of triazolam, increasing its plasma concentration, cimetidine and erythromycin inhibiting hepatic metabolism of triazolam with an increase of plasma concentration and a retarded clearance [24,26]. Alcohol consumption, cigarette smoking may decrease sedative effects of concurrent benzodiazepines or administration with other CNS depressants resulting with additive CNS depression and a greater risk of apnea. Respiratory arrest and depression has been reported in patients receiving benzodiazepines and clozapine simultaneously. Therefor in order to avoid toxicological events with benzodiazepines should be considered the drugs which are used simultaneously with them [21,22].

1.3.3. Antidote for benzodiazepine toxicity

Flumazenil is a drug used for elimination and cure of benzodiazepines toxicity, it acts to reverse the effect of benzodiazepines. Flumazenil is used in order to induce benzodiazepines sedation, which has been cause from overdose. However, flumazenil usage can be controversial in patients who has developed chronic tolerance of benzodiazepines use or abuse, and therefore result in withdrawal seizures, as well as may not result in reverse respiratory depression which has been caused by the overdose [30]. Flumazenil is recommended antidote for patients who are not using benzodiazepines chronically, and shows to be safe and effective. Usually is administered at 0,2 mg and injected intravenously, and can reach up to a maximum dose of 1 mg until desirable effect occurs [33]. It can be re-injected in event of re-sedation, but not more than 3 mg should be given to the patient within 1 hr. Flumazenil duration is from 0,7 to 1,3 hrs, therefore may not completely exceed some benzodiazepines that are long acting or administered at high doses, consequently used at higher doses, some may need continuous flumazenil infusion. Up to 1 mg for 1hr [24,25]. On the other hand, naloxone can be recommended at very low doses if the diagnosis is not clear or an opiate co-ingestion has been observed. Charcoal can be recommended for symptomatic patients for GI decontamination or assisted ventilation produce for respiratory depression [30,31,32]
1.4. Alprazolam

Alprazolam is a triazolo-benzodiazepine and used in the treatment of various central nervous system disorders such as generalized anxiety, panic attacks or depression. [3] In Lithuania alprazolam authorized as medicines, "Alprasedon, Alprazolam - Grindeks, Alrazolam - Orion", "Xanax", Zomiren. [2]

\[ C_{17}H_{13}ClN_4 \]  MR 308.8

IUPAC: 8-Chloro-1-methyl-6-phenyl-4H- [1,2,4] triazolo [4,3-a] [1,4] benzodiazepine [3]

Chemical-Physical Characteristics, solubility and etc.

Alprazolam is a white or almost white, crystalline powder. Practically insoluble in water, soluble in chloroform and dichloromethane, poorly soluble in acetone and ethanol [33].

Pharmacological properties

It is a lipophilic substance which readily penetrates the blood-brain barrier and reach the central nervous system. It connects to the high affinity of gamma aminobutyric acid (GABA) receptors, alprazolam increases the chlorine ion channel opening frequency causing hyperpolarization of nerve cells [34,35]. Alprazolam pharmacological effects is dose dependent. Therapeutics inhibits anxiety, and has sedative and hypnotic effect. In large doses it causes amnesia, provokes seizures and relaxes the skeletal muscles.

Elimination

Alprazolam is well absorbed from the gastrointestinal tract - depending on the dose up to 90% of the administered amount reaches the systemic circulation. Single doses of 1 mg alprazolam dose maximum volume (Cmax) of 12-22 g / l is reached after 12 hours (Tmax). About 80% of alprazolam blood circulates bound to serum proteins [36,37]. Alprazolam classified as a medium-term effects medication in the benzodiazepines group. Its half-life, depending on the individual patient's metabolism, ranging from 6.3 to 25.9 hours (t1 / 2). [35,36,] The volume of distribution (Vd) is 0.8 to 1.3 L / kg and clearance (Cl) ranges from 0.7 to 1.5 ml / min / kg. Older patients with a lower clearance compared to younger patients, and require dose adjustment [39].

Ways of Metabolism
Alprazolam is metabolized in the liver by the cytochrome P450 system. The two main metabolites 4-hydroxy alprazolam and α- hydroxy alprazolam has weak effect compared to the non-metabolized drug. Up to 80% of alprazolam through the kidney is removed as non-metabolized form. With withdrawal prolongs renal and hepatic pathologies, obesity [36,40].

1.5. Estazolam

Estazolam is triazolo-benzodiazepine. It is commonly prescribed for short-term treatment of insomnia. [2,48]. In Lithuania estazolam authorized as medicines, "Estazolam TC” [2].

\[
\begin{align*}
\text{C}_{16}\text{H}_{12}\text{N}_4 
\end{align*}
\]

IUPAC: 8-chloro-6-phenyl-4H-[1,2,4]triazolo [4,3-a][1,4]benzodiazepine[3]

**Chemical-physical characteristics, solubility and etc.**

Estazolam is white crystalline powder. Practically not soluble in water, and soluble in ethanol.

**Pharmacological properties**

Estazolam binds to the gamma-aminobutyric acid (GABA) receptor at a site distinct from the inhibitory neurotransmitter GABA binding site in the limbic system of the central nervous system (CNS). This binding leads to an opening of the chloride channels, which allows the flow of chloride ions into the neuron, hyperpolarizes the neuronal membrane and stabilizes it, which is characterizes by less excitable state leading to inhibition of neuronal firing, and resulting in decrease neuronal excitability. Estazolam is binding to GABA-A receptors [42].

**Elimination**
Estazolam is well absorbed from the gastrointestinal tract - depending on the dose up to 93% of the administered amount reaches the systemic circulation. Single doses of 1 mg or 2 mg of estazolam dose maximum volume (Cmax) is 55 to 98 ng / ml is reached after 2 hours (0.5 to 6 hours) (Tmax). About 93% of alprazolam blood circulates bound to serum proteins. [43,41] Estazolam classified as intermediate-acting oral benzodiazepines. Its half-life, depending on the individual patient metabolism between 10 and 24 hours (t1 / 2) average 17 hours [44].

Ways of Metabolism

Estazolam metabolized by the liver cytochrome P450 3A (CYP3A). The two main metabolites 4-hydroxy-estazolam is a key metabolite in the serum and the second metabolite (1-oxo-estazolam). The main route of excretion is via the kidney [46]. After 5 days, about 87% of the administered radioactivity was excreted in human urine. Following oral administration of 2 mg of the medicinal product, about 5% of the dose is excreted unchanged by the kidneys, and 4% - feces. Most of the oral dose is excreted via the kidneys as 4-hydroxy-estazolam and 1-oxo-estazolam form. Factors such as renal and hepatic pathologies, as well as obesity can influence on excretion and elimination of the drug [47].

1.6. Triazolam

Triazolam is a triazolo-benzodiazepine and is used in the treatment of various central nervous system disorders such as generalized anxiety, panic attacks or depression, but mainly short term insomnia (7 to 10 days) [3]. In Lithuania triazolam is authorized as medicinal preparations such as : Halcion [2]

\[ C_{17}H_{12}C_{12}N_{4} \text{ M}_{R} 343.2 \]

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

**Chemical-physical characteristics, solubility and etc.**

A white or pale yellow crystalline powder. Practically insoluble in water and ether; soluble alcohol, chloroform; hydrochloric acid.
Pharmacological properties

Triazolam is a triazolo-benzodiazepine derivative with sedative-hypnotic property. A short-acting benzodiazepine used as a hypnotic agent in the treatment of insomnia. Triazolam is nowadays dispensed from the market due to extensive adverse drug reaction observed, due to high dosage. Triazolam interacts mainly with GABA-a which stimulates the chloride channels to open on the neural membrane. This results in hyperpolarization and synaptic inhibition reaching ultimate decrease in neuronal excitability [49,50].

Elimination

Triazolam rapidly and is nearly completely absorbed from the GI tract - depending on the dose to 90% of the amount consumed is absorbed systemically. Single doses of 1 mg alprazolam dose maximum volume (Cmax) 1-6 ng / ml is reached 2 hours after administration (Tmax). About 89% of triazolam in blood circulates bound to serum proteins [3,50,51]. Triazolam medium-term effects of benzodiazepines group. Its half-life, depending on the individual patient metabolism and ranges from 1.5 to 5.5 hours (t1 / 2) [52]. The volume of distribution (Vd) is 0.6 to 1.7 L / kg. Older patients needs to get dose adjustment.

Ways of Metabolism

Triazolam undergoes hepatic microsomal oxidation to inactive hydroxylated metabolite via CYP3A4, which primarily are eliminated as glucuronide to 6 conjugates. It has a biphasic half-life, with a mean reported apparent half-life of 3.4 hr for the initial phase and 7.8 hr for the terminal phase. Elimination half-life for triazolam is 1.5-5.5 hr. Triazolam and its metabolites, principally as conjugated glucuronides, which are inactive, are mainly excreted in the urine. Some amount are excreted as un-metabolized triazolam. The two primary metabolites accounted for 79.9% of urinary excretion [51,53].

1.7. Triazolam, estazolam and alprazolam qualitative evaluation using 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 solvents 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) [54].

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

Chromatography in a thin layer of sorbent (TLC) is widely used for screening benzodiazepines and their metabolites. TLC methodic is recommended for identification of a large number of substances, which allows to notice the substance in a relatively small amount of time, therefor 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. On the other hand, it may provide some differential data results from the other methodic present, 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 [55,56].

1.7.2. Thin-layer-chromatography methodics suitable for triazolam, estazolam and alprazolam identification

The scientific literature contains various analysis by thin layer chromatography methods which may be used for alprazolam, estazolam and triazolam identified 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 4,5,6 provide information on the literature for thin layer chromatography techniques, which can be adapted for quality assessment of alprazolam, estazolam and triazolam.

There was identified a multiple of methodics in the literature suitable for determination of alprazolam, triazolam, and estazolam. Benzodiazepines form a diverse group of chemicals; however, the following thin-layer chromatographic systems, when used in combination, give good separations for a number of benzodiazepines:

According to UNODC “Recommended methods for the identification and analysis of
barbiturates and benzodiazepines’’ [57,58,59,60]. The three suitable systems were:

**Table 4. Rf index for alprazolam, estazolam, triazolam according to UNODC methods.**

<table>
<thead>
<tr>
<th>System</th>
<th>Solvent system</th>
<th>Alprazolam Rf values</th>
<th>Estazolam Rf values</th>
<th>Triazolam Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Chloroform - acetone (80:20, v/v)</td>
<td>0,01</td>
<td>0,08</td>
<td>0,62</td>
</tr>
<tr>
<td>B</td>
<td>Chloroform -methanol (90:10 v/v)</td>
<td>0,01</td>
<td>0,11</td>
<td>0,50</td>
</tr>
<tr>
<td>C</td>
<td>Cyclohexane – toluene- diethylamine (75:15:10 v/v/v)</td>
<td>1,0</td>
<td>0,09</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 Acidified potassium iodoplantate reagent.

According to Clark’s analysis [61]:

**Table 5. Rf index for alprazolam, estazolam, triazolam according to Clark’s analysis.**

<table>
<thead>
<tr>
<th>System</th>
<th>Solvent systems</th>
<th>Alprazolam Rf values</th>
<th>Estazolam Rf values</th>
<th>Triazolam Rf values</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>Methanol–25% ammonia (100:1.5)</td>
<td>0.67</td>
<td>-</td>
<td>0.60</td>
</tr>
<tr>
<td>TB</td>
<td>Cyclohexane–toluene–diethylamine (15:3:2)</td>
<td>0.01</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>TC</td>
<td>Chloroform–methanol (9 : 1)</td>
<td>0.57</td>
<td>-</td>
<td>0.40</td>
</tr>
<tr>
<td>TD</td>
<td>Chloroform–acetone (4 : 1)</td>
<td>0.07</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>TE</td>
<td>Ethyl acetate–methanol–25% ammonia (17:2:1)</td>
<td>0.47</td>
<td>-</td>
<td>0.44</td>
</tr>
<tr>
<td>TF</td>
<td>Ethyl acetate</td>
<td>0.02</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>TL</td>
<td>Acetone</td>
<td>0.14</td>
<td>-</td>
<td>0.16</td>
</tr>
<tr>
<td>TAD</td>
<td>Chloroform–methanol (9 : 1)</td>
<td>0.14</td>
<td>-</td>
<td>0.41</td>
</tr>
<tr>
<td>TAE</td>
<td>Methanol</td>
<td>0.67</td>
<td>-</td>
<td>0.68</td>
</tr>
<tr>
<td>TAF</td>
<td>Methanol–n-butanol (3 : 2) containing 0.1 mol/L sodium bromide</td>
<td>0.66</td>
<td>-</td>
<td>0.65</td>
</tr>
</tbody>
</table>
According to Clark’s analysis, 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 appears, Dragendorff – yellow, orange or brown orange spots, acidified iodoplplatinate solution giving violet, blue-violet coloured spots. Location reagents for systems TD, TE and TF: Van Urk’s reagent, Ferric chloride, Mercurous nitrate, Acidified potassium permanganate, Furfuraldehyde reagent, Acidified iodoplplatinate solution.

According to: Thin layer chromatographic separation of benzodiazepines derivate [62].

**Table 6. Rf index for alprazolam according Thin layer chromatographic separation of benzodiazepines derivate**

<table>
<thead>
<tr>
<th>System</th>
<th>Solvent system</th>
<th>Alprazolam Rf value</th>
<th>Estazolam Rf value</th>
<th>Triazolam Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ethyl acetate</td>
<td>0,19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>Chloroform-methanol (9:10)</td>
<td>0,42</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>Chloroform-acetone(4:1)</td>
<td>0,18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>Ethyl acetate-methanol-ammonia (17:2:1)</td>
<td>0,57</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>Hexane –chloroform- methanol (5:5:1)</td>
<td>0,2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VI</td>
<td>Acetone-Chloroform-isopropanol (8:1:1)</td>
<td>0,4</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

According to Thin layer chromatographic separation of benzodiazepines derivatives, it has been applied 5µl of a 2mg/ml sol in methanol for determination. The chambers were saturated; stationary phase was silica gel GF254. Visualization was performed with UV light (both 254 and 350 nm). Location reagent used Drangendorff.

The tables show that most of the solvent systems described is not suitable for alprazolam, estazolam and triazolam mixture separation. Frequently, in case of separation is not possible because of the close Rf values or solvent system adequacy has not been evaluated for all three materials. As TLC mobile phase components often used methanol, cyclohexane, toluene, acetone, ammonium
hydroxide and diethylamine. While analysing the literature it has not been found any information about alprazolam, estazolam and triazolam mixture qualitative assessment by thin layer chromatography, and it was concluded that the optimal mixture testing mobile phase will be sought experimentally.

1.8. **Triazolam, estazolam and alprazolam 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 chromatography is mainly stated on mechanisms of absorption, mass distribution, ion exchange, size exclusion or stereo-chemical interaction. [54]

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. [63]

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 [64,65].

HPLC has shown his advantages due to 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. Therefor HPLC offers an attractive analytical alternative for the routine determination of 1,4-benzodiazepines in biological samples bellow [64,65,66].
1.8.2. High-performance liquid chromatography methodologies suitable for triazolam, estazolam and alprazolam identification

Scientific literature has described various methods for the HPLC, which was to investigate the alprazolam, codeine or paracetamol. They are widely used drug in clinical trials assessing the pharmacokinetic properties of the materials and the industry in quality control. Table 7 shows methodologies that can be used for alprazolam, estazolam and triazolam for qualitative analysis in pharmaceutical preparations.

Table 7. HPLC methodologies suitable for alprazolam, triazolam qualitative analysis according to Clark’s analysis [62]:

<table>
<thead>
<tr>
<th>Methodics</th>
<th>Alprazolam RT</th>
<th>Triazolam RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>HY</td>
<td>-</td>
<td>4.2 min</td>
</tr>
<tr>
<td>HI</td>
<td>4.70 min</td>
<td>4.38 min</td>
</tr>
<tr>
<td>HJ</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HK</td>
<td>2.79 min</td>
<td>1.83 min</td>
</tr>
<tr>
<td>HAA</td>
<td>4.0 min</td>
<td>17.4 min</td>
</tr>
<tr>
<td>HAX</td>
<td>6.4 min</td>
<td>6.4 min</td>
</tr>
<tr>
<td>HAY</td>
<td>6.4 min</td>
<td>6.7 min</td>
</tr>
</tbody>
</table>

System HY:
- Column: C18 symmetry (250 4.6 mm i.d., 5 mm), with column temperature 40 C.
- Mobile phase: (A:B) sulfuric acid (0.5 mL 2.5 mol/L) in water(500 mL)–sulfuric acid (0.5 mL 2.5 mol/L) in acetonitrile (500 mL).
- Detector: DAD
- Elution Program: (98 : 2) for 3 min to (2 : 98) over 23 min, hold for 10 min, back to initial conditions over 2 min with equilibration of 8 min before next injection

System HI:
- Column: ODS Hypersil (200x5mm i.d., 5 mm)
- Mobile phase: Methanol–water–phosphate buffer
- Detector: DAD
- Elution Program: (55:25:20)

System HJ:
- Column: ODS Hypersil (200x5mm i.d., 5 mm)
- Mobile phase: Methanol–water–phosphate buffer
- Detector: DAD
- Elution Program: (70:10:20)

System HK:

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

System HAA:

- Column: C8 Symmetry (250 4.6 mm i.d., 5 mm) with Symmetry C18 precolumn (20 mm)
- Mobile phase: (A:B) phosphate buffer (pH 3.8)–acetonitrile.
- Detector: DAD
- Column tempertaure: 30°C.
- Elution Program: (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.
- Flow rate: 1 mL/min

System HAX:

- Column: Supelcosil LC-DP (250 4.6 mm i.d., 5 mm).
- Mobile phase: (A : B : C) Acetonitrile–phosphoric acid (0.025%v/v)–triethylamine buffer.
- Detector: DAD (λ229 nm)
- Elution Program: Isocratic elution: (25 : 10 : 5)
- Flow rate: 0.6 mL/min.

System HAY:

- Column: LiChrospher 100 RP-8 (250 4.0 mm i.d., 5 mm)
- Mobile phase: (A : B : C) Acetonitrile–phosphoric acid (0.025%v/v)–triethylamine buffer.
- Detector: DAD (λ 229 nm)
- Elution Program: Isocratic elution: (60 : 25 : 15)
- Flow rate: 0.6 mL/min.

According to UNODC “Recommended methods for the identification and analysis of barbiturates and benzodiazepines”. [67,68,69,70,71]. The two suitable systems were:
Table 8. HPLC methodics suitable for alprazolam, triazolam qualitative analysis.

<table>
<thead>
<tr>
<th>Methodics</th>
<th>Alprazolam RT</th>
<th>Estazolam RT</th>
<th>Triazolam RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3,35</td>
<td>4,22</td>
<td>5,18</td>
</tr>
<tr>
<td>B</td>
<td>1,28</td>
<td>1,08</td>
<td>1,13</td>
</tr>
</tbody>
</table>

System A:
- Column: C18 symmetry (250 4.6 mm i.d., 5 mm)
- Mobile phase: Methanol: water: phosphate buffer (0.1M)
- Detector: DAD
- Elution Program: (55:25:20, v/v/v), pH 7.25
- Flow rate: 1.5 ml/min

System B:
- Column: C18 symmetry (250 4.6 mm i.d., 5 mm)
- Mobile phase: Methanol: water: phosphate buffer (0.1M)
- Detector: DAD
- Elution Program: (70:10:20, v/v/v), pH 7.6
- Flow rate: 1.5 ml/min

The analysis of the literature found studies that analysed alprazolam and triazolam, however it has been very hard to seek sources for estazolam analysis. Table 7 and 8 shows the HPLC methodology adapted to analyze all three pharmaceutical preparations separately, but the pharmaceutical’s retention times in many systems are very close, so the mixture analysis described methods are not adequate. HPLC literature methodic were used as a base for the establishment and development of appropriate HPLC methodology suitable for alprazolam, estazolam and triazolam qualitative analysis when they are 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 alprazolam, estazolam and triazolam were studied. Materials have been extracted from “Xanax”, Estazolam TC, “Halcion 250 µg”.

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 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 a more qualitative and accurate results - glass plates. The standard and tested solution has 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 standard solution have been prepared using methanol solvent.

For preparation of mobile solvent system such solvents were used as: toluene, cyclohexane, methanol, diethylamine, triethylamine, ethyl acetate, chloroform.

Visualizer

Dried chromatographic plates are observed with:

- UV light (254 nm). Further obtained spots of mixture prepared from medical substances are identified for Rf values using the imaging device CAMAG TLC Visualizer connected with a software VideoSCan equipment.
- Dragendorff reagent (modified according Munja), which is prepared 0.85 g of bismuth nitrate (Bi(NO₃)₃) dissolving in 70 ml of purified water and 10 ml of glacial acetic acid (CH₃COOH) solution. 8.0 g of potassium iodide (KI) was dissolved in 20 ml of purified water. The solutions are mixed. The developing agent is a solution obtained by mixing 5 ml of the mixture with 10 ml of glacial acetic acid and by diluting it with purified water to 50 mL.

**Standard solutions**

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

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

**Test solutions**

Solution for analysis have been prepared from the drugs obtained in the pharmacy stores in Lithuania. Alprazolam solution (A1) was produced from medicinal product “Xanax” Pfizer H.C.P, which are 1 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to volumetric flask and dissolve 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.

Estazolam standard solution (E1) has been prepared from medicinal product “Estazolam TC” (Polfa Tarchomin), which are 2 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to volumetric flask and dissolve 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 20 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 subsequence researches.

Triazolam standard solution (T1) has been prepared from medicinal product “Halcion 250 µg” (Pfizer H.C.P), which are 250 µg dosage tablets. 2 tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to 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 – RSA 1, RSE 1, RST 1 and their mix RS MAET 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 CAAMAG Linomat5 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 of 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.

\[
R_f = \frac{L_1 (mm)}{L_2 (mm)}
\]

The experimental analysis is to determine the most appropriate solvent system for separation of alprazolam, estazolam and triazolam mixtures. Therefore, in order to find the most appropriate methodology it has been carried multiple studies with separate solutions, the reference solutions (RSA 1, RSE 1, RST 1), the tested solutions (A1, E1, T1) and the subsequent mixture solution (RSMAET 1, MAET) 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 (254nm). 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 has been acquired using chromatograph (Waters 2695) with a photodiode array detector (Waters 996, at wavelength 200-400nm range). Mixtures solution separation has 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 has 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 solution of concentration of 0,1 mg/ml: reference solution alprazolam (Sigma–Aldrich, JAV) estazolam (Sigma–Aldrich, JAV), triazolam (Sigma–Aldrich, JAV). Reference solutions: alprazolam RSA, estazolam RSE, triazolam RST.

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

Test solutions

Solution for analysis have been prepared from the drugs obtained in the pharmacy stores in Lithuania. Alprazolam solution (A2) was produced from medicinal product “Xanax” (Pfizer H.C.P), which are 1 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to 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.
Estazolam standard solution (E2) has been prepared from medicinal product “Estazolam TC” (Polfa Tarchomin), which are 2 mg dosage tablets. The tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to volumetric flask and dissolve 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 medicinal product “Halcion 250 µg” (Pfizer H.C.P), which are 250 µg dosage tablets. 2 tablet has been crushed and grinded well in the mortar. The powder obtained has been transferred to 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 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 alprazolam, estazolam, 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 0,1mg/ml and therefore has been analyzed with high performance liquid chromatography under optimized conditions. Alprazolam, estazolam, triazolam mixture RS MAET2 made from standard solutions for analysis with HPLC methodic required to optimize the composition of the eluent and the elution method (isocratic or gradient mode). However, the injection mode remained constant 10 µl. Alprazolam, estazolam, 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 alprazolam, estazolam and triazolam medicinal product.
3. RESULTS AND DISCUSSION

3.1. Thin-layer chromatography (TLC) methodic selection

Visualizer selection

TLC optimization methodology for separation and identification of alprazolam, estazolam and triazolam in their mixture, in particular was chosen appropriate developing reagent. After the tests, in order to assess which visualizer is good for all three substances identified, were singled out two spots in developing methods: Spraying Dragendorff reagent (modified by Munja) lighting or UV light (254 nm, 365nm). In both cases, it was not shown clear medicinal substances spots chromatography plate, only by determining it with the UV light. (Fig.4 and Fig. 5)

**Fig.4 Alprazolam (RS A1), triazolam (RS T1), estazolam (RS E1) spots on the chromatographic plate observed under UV light (254nm, 365 nm) SS-1**

**Fig.5 Alprazolam (RS A1), triazolam (RS T1), estazolam (RS E1) spots on the chromatographic plate sprayed over with Dragendorff reagent**

Spraying of the pharmaceutical solutions tested stains on a chromatographic plate with Dragendorff reagent (modified according Munja) alprazolam, estazolam and triazolam appears to be absent (Figure 5). Compared with the developing agent to UV light, this method makes it possible to identify not only according to Rf values of reference solution, but also by color reaction with Dragendorff reagent.
Illuminating the chromatography plate under UV light (254 nm; 365 nm) revealed gently purple stain of test substances (Figure 4). This way of visualizing, comparing it with the washer Dragendorff reagent is cheaper, easier to apply and less determined by investigating personal contact with the active chemical reagents.

**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 alprazolam, estazolam and triazolam and their mixtures subsequently. In order to find the most suitable, a numerous number of solvents has been used, and their ratio composition has been adjusted to therefor find the most appropriate solvent system. According to the reference solution it has been identified the right and approved systems. And has been selected 5 most fitting SS system suitable for appearing separation of the medicines. (Table 9)

*Table 9. Average alprazolam, estazolam and triazolam Rf in different solvent systems.*

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Alprazolam av. Rf</th>
<th>Estazolam av. Rf</th>
<th>Triazolam av. Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclohexane - methanol- diethylamine (75:15:10) (SS – 1)</td>
<td>0,42</td>
<td>0,38</td>
<td>0,35</td>
</tr>
<tr>
<td>Toluene - methanol - diethylamine (75:15:10) (SS – 2)</td>
<td>0,32</td>
<td>0,27</td>
<td>0,28</td>
</tr>
<tr>
<td>Chloroform – methanol-diethylamine (75:15:10) (SS – 4)</td>
<td>0,18</td>
<td>0,12</td>
<td>0,11</td>
</tr>
<tr>
<td>Chloroform- Methanol (90:10)(SS – 5)</td>
<td>0,08</td>
<td>0,02</td>
<td>0,01</td>
</tr>
<tr>
<td>Cylohexane – Toluene – diethylamine - methanol (70:10:10:10) (SS – 6)</td>
<td>0,25</td>
<td>0,21</td>
<td>0,20</td>
</tr>
</tbody>
</table>

**Statistic evaluation**

For investigation and assessment of the reliable methodic and system chosen, we have performed analysis of mix solutions (RS MAET) 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, relative error at a confidence level of 0.95, as well as a confidence interval when the error $p=0.05$ (table 8)

*Table 10. Statistical test results and Rf values using a SS-1, SS-2, SS-3, SS-4, SS-5 evaluating systems for analysed medicinal preparations.*

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Tested solution</th>
<th>Rf average</th>
<th>Standard deviation</th>
<th>Error</th>
<th>Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS – 1</td>
<td>Alprazolam</td>
<td>0.42</td>
<td>0.006</td>
<td>0.003</td>
<td>0.41 - 0.42</td>
</tr>
<tr>
<td></td>
<td>Estazolam</td>
<td>0.38</td>
<td>0.008</td>
<td>0.004</td>
<td>0.36 - 0.38</td>
</tr>
<tr>
<td></td>
<td>Triazolam</td>
<td>0.35</td>
<td>0.007</td>
<td>0.003</td>
<td>0.33 - 0.35</td>
</tr>
<tr>
<td>SS – 2</td>
<td>Alprazolam</td>
<td>0.32</td>
<td>0.005</td>
<td>0.003</td>
<td>0.30 - 0.32</td>
</tr>
<tr>
<td></td>
<td>Estazolam</td>
<td>0.27</td>
<td>0.008</td>
<td>0.004</td>
<td>0.27 - 0.28</td>
</tr>
<tr>
<td></td>
<td>Triazolam</td>
<td>0.28</td>
<td>0.009</td>
<td>0.004</td>
<td>0.28 - 0.30</td>
</tr>
<tr>
<td>SS – 3</td>
<td>Alprazolam</td>
<td>0.18</td>
<td>0.007</td>
<td>0.003</td>
<td>0.18 - 0.20</td>
</tr>
<tr>
<td></td>
<td>Estazolam</td>
<td>0.12</td>
<td>0.004</td>
<td>0.002</td>
<td>0.11 - 0.12</td>
</tr>
<tr>
<td></td>
<td>Triazolam</td>
<td>0.12</td>
<td>0.004</td>
<td>0.002</td>
<td>0.12 - 0.12</td>
</tr>
<tr>
<td>SS – 4</td>
<td>Alprazolam</td>
<td>0.08</td>
<td>0.008</td>
<td>0.005</td>
<td>0.08 - 0.09</td>
</tr>
<tr>
<td></td>
<td>Estazolam</td>
<td>0.02</td>
<td>0.004</td>
<td>0.002</td>
<td>0.01 - 0.02</td>
</tr>
<tr>
<td></td>
<td>Triazolam</td>
<td>0.01</td>
<td>0.004</td>
<td>0.002</td>
<td>0.01 - 0.01</td>
</tr>
<tr>
<td>SS – 5</td>
<td>Alprazolam</td>
<td>0.25</td>
<td>0.005</td>
<td>0.002</td>
<td>0.24 - 0.26</td>
</tr>
<tr>
<td></td>
<td>Estazolam</td>
<td>0.21</td>
<td>0.006</td>
<td>0.003</td>
<td>0.19 - 0.21</td>
</tr>
<tr>
<td></td>
<td>Triazolam</td>
<td>0.20</td>
<td>0.007</td>
<td>0.004</td>
<td>0.19 - 0.20</td>
</tr>
</tbody>
</table>
As we can see from the diagram above (Diagram 1), systems: SS-2, SS-3, SS-4, SS-5 cannot be valid for analysis of medicinal preparations, because of very closely related Rf values of estazolam and triazolam, which accounts of <0,02 Rf difference between the values of the two medicinal products. SS-4 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 alprazolam, estazolam 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 (alprazolam and estazolam) was > 0,04 (alprazolam and triazolam) > 0,07, and (estazolam and triazolam) > 0,03.
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 have been performed on the medicinal preparations obtained from Lithuanian pharmacies. Analysis have been performed on the SS-1 and visualizing with UV light (254nm)

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

<table>
<thead>
<tr>
<th>Solvent system</th>
<th>Alprazolam</th>
<th>Estazolam</th>
<th>Triazolam</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RS A1</td>
<td>RS E1</td>
<td>RS T1</td>
</tr>
<tr>
<td>SS – 1</td>
<td>0,41</td>
<td>0,38</td>
<td>0,34</td>
</tr>
</tbody>
</table>

Fig. 6 SS-1 under UV light (254nm) analysis performed on reference alprazolam (RS A1), estazolam (RS E1), triazolam (RS T1) and their mix (RS MAET1)
Analysed preparations extracted from pharmaceuticals shows that the Rf values obtained during analysis fits into the confidence interval set to the reference solutions, therefor meets the requirements for solvent system SS-1, Alprazolam obtained Rf =0,41 confidence interval (0,41-0,42), estazolam obtained Rf=0,38 with confidence interval (0,36 -0,38) and triazolam obtained Rf=0,34 with confidence interval (0,33-0,35) therefor can be concluded that SS-1 is valid for qualitative analysis of alprazolam, estazolam 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. Therefor a qualitative evaluation of the mixtures can be performed. Accounting this criteria, 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 (RSAET 2) were observed using column: Sunfire C18 (length 15 cm, internal diameter 3.0 mm, sorbent particle size 3,5µm) Supelco LC18 (length 15cm, inner diameter 4,6mm; resin particle size of 5,0µm) and an ACE C18 (length 25cm, inner diameter 4.6 mm, the resin particle size 5µm). Isolation best achieved through the ACE C18 chromatography (25cm × 4mm × 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 (RSAET2) 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.

Alprazolam, estazolam and triazolam mixtures, 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 the Table 12).

*Table12. 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>00:00</td>
<td>1,0</td>
<td>98,0</td>
<td>2,0</td>
</tr>
<tr>
<td>1</td>
<td>1,0</td>
<td>98,0</td>
<td>2,0</td>
</tr>
<tr>
<td>20</td>
<td>1,0</td>
<td>2,0</td>
<td>98,0</td>
</tr>
<tr>
<td>23</td>
<td>1,0</td>
<td>2,0</td>
<td>98,0</td>
</tr>
<tr>
<td>24</td>
<td>1,0</td>
<td>98,0</td>
<td>2,0</td>
</tr>
<tr>
<td>29</td>
<td>1,0</td>
<td>98,0</td>
<td>2,0</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 1,0 ml/min. General chromatographic analysis duration was 29 minutes.
3.3.1 Adapted HPLC methodic validation

High performance liquid chromatography method was validated according to selected settings – precision, linearity, limit of detections and limit of determination.

Specificity

Method of specificity is a 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.

1. Chromatogram of the sample mixtures released from the drug preparations. (Fig.8) We can see that alprazolam retention time is 13,216 min, estazolam – 13,407 min, and triazolam – 14,340 min.

![Chromatogram](image)

*Fig.8. Tablet extract chromatogram 1 – Alprazolam (retention time 13,216 min.), 2 – Estazolam (13,407) – Triazolam (14,340)*

2. Comparison of the UV light spectrum absorbance’s chromatogram to the absorption spectrum in literature (Fig 9, Fig 10, Fig 11).
Fig. 9 Alprazolam absorbance spectrum a) obtained by measurement with DAD detector, b) obtained from "Clarke’s Analysis of Drug and Poisons"

Fig. 10 Estazolam absorbance spectrum a) obtained by measurement with DAD detector, obtained from Japanese Pharmacopoeia 16th edition

Fig. 11 Triazolam absorbance spectrum a) obtained by measurement with DAD detector, b) obtained from "Clarke’s Analysis of Drug and Poisons"

3. Chromatogram obtained from standard solution.

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 in 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 are performed on two different days.

We can see the results in (table 13) which is measured by (RSD) relative standard deviation, also known as coefficient of variation. In this quantitative research method in the first day should be ≤5 percent, and the second day ≤10 percent. RSD is the percentage of the average peak ratio and the standard deviation.

Table 13. Test solution repeatability coefficient according to variation of the retention time and peak area

<table>
<thead>
<tr>
<th>Tested solution</th>
<th>RSD results</th>
<th>First day</th>
<th>Second day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Area</td>
<td>Ret.time</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>2,5</td>
<td>0,052</td>
<td>9,0</td>
</tr>
<tr>
<td>Estazolam</td>
<td>3,0</td>
<td>0,051</td>
<td>8,5</td>
</tr>
</tbody>
</table>
Triazolam | 3,5 | 0,026 | 5,2 | 0,028

Based on the data obtained showed that RSD analyte retention time and peak width does not exceed 5 percent. On the same day and did not exceed 10 percent. tests between a few days. As a result, it can be said that the method is suitable for alprazolam, triazolam and estazolam qualitative and quantitative analysis.

**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. Alprazolam calibration curve (fig.14) is composed out of 6 points, respectively estazolam (fig. 13) and triazolam (fig.15) as well. The resulting characteristics are shown in the (table 13). Obtained results are 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>Alprazolam</td>
<td>0,9999797</td>
<td>f(x)=1.08298e+007*x+1989.43</td>
<td>0.000937 - 0.03</td>
</tr>
<tr>
<td>Estazolam</td>
<td>0,9999596</td>
<td>f(x)=1.41288e+007*x+5012.14</td>
<td>0.000937 - 0.03</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0,9999613</td>
<td>f(x)=1.13786e+007*x+3775.44</td>
<td>0.000937 - 0.03</td>
</tr>
</tbody>
</table>
Fig. 13 Estazolam calibration curve, calibration curve equation: 
\[ f(x)=1.41288 \times 10^7 x + 5012.14 \]

Fig. 14 Alprazolam calibration curve, calibration curve equation: 
\[ f(x)=1.08298 \times 10^7 x + 1989.43 \]

Fig. 15 Triazolam calibration curve, calibration curve equation: 
\[ f(x)=1.13786 \times 10^7 x + 3775.44 \]
**LOD and LOQ limits**

*LOD (limit of detection)* is considered the calculation of the lowest mount possible of the analyte which can be observed, but not obligatory quantified as an exact value. Usually is calculated based on the known concentration of analyzed analyte and further established the minimum level at which the analyte can be detected.

*LOQ (limit of quantification)* is the smallest amount of the analyte which is reliably easy to determine in sample with precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly 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>Alprazolam</td>
<td>0,01</td>
<td>0,022</td>
</tr>
<tr>
<td>Estazolam</td>
<td>0,012</td>
<td>0,025</td>
</tr>
<tr>
<td>Triazolam</td>
<td>0,020</td>
<td>0,045</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 alprazolam, estazolam and triazolam in the mixture, the methodology was applied in analyzing solutions A2, E2 and T2 made of pharmaceutics medicines from pharmacies located in Lithuania.

Examination of medicinal product "Xanax" solution, alprazolam in the chromatogram were identified by the following criteria: a retention time (13,382), which is close to the reference solution for a retention time of alprazolam; UV light absorption spectrum that is identical to the literature alprazolam UV absorption spectrum (Fig 16)
Analyzing medicinal product “Estazolam TC”, estazolam was identified on the chromatogram with a retention time 13.204 min and a UV light absorption spectrum that is identical to the literature UV absorption spectrum. (fig.17)

Analyzing medicinal product Halcion, triazolam was identified on the chromatogram with a retention time 14.330 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. Alprazolam (Fig.14), Estazolam (Fig.13.), and Triazolam (Fig.15) calibration curve made from 6-point concentration range of - 0.000937 mg / ml - 0.03 mg/ml.

Fig. 18 Analyzed medicinal solution T2 (triazolam)
4. CONCLUSION

1. In analysis of literature it has been not found any methodic which would be suitable for separation of mixture solutions containing medicinal product: alprazolam, estazolam, triazolam for thin layer chromatography. But thought out 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: alprazolam (Rf=0,41), estazolam (Rf=0,38) and triazolam (Rf=0,34). Upon repetition of analysis, it has been stated that the Rf value haven’t exceeded repetition error p <0,5 limitation. Chosen TLC methodic can be concluded to be acceptable for qualitative analysis of alprazolam, estazolam and triazolam.

2. In analysis of literature it has been not found any methodic which would be suitable from the separation of mixture solutions containing medicinal product: alprazolam, estazolam, triazolam for high performance liquid chromatograph for quality assessment. But through experimental work alprazolam, estazolam, 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 injection volume of 10 μl. Medicines have been identified with help of diode array detector. After performing analysis with reference solution, analysis with medicinal mixture has been examined and have stated the retention time: alprazolam (13,216 min), estazolam (13,407 min) and triazolam (14,340 min). Retention time upon repetition of analysis have not exceeded the relative error of p <0,05 limitation.

3. When the optimum conditions for analysis by thin layer chromatography has been adjusted, the selected methodic has been applicable for the medicinal preparation of “Xanax” (Pfizer H.C.P), “Estazolam TC” (Polfa Tarchomin), “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, was concluded that thin layer chromatography method was suitable to separate alprazolam, estazolam, triazolam pharmaceutical preparations.

4. Chosen high performance liquid chromatography methodology was proven to be suitable for the medicinal preparation of “Xanax” (Pfizer H.C.P), “Estazolam TC” (Polfa Tarchomin), “Halcion” (Pfizer H.C.P) qualitative assessment. Alprazolam, estazolam and triazolam medicinal products have been established in accordance to:
   - 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. Chosen method is suitable for separation of alprazolam, estazolam an triazolam qualitative assessment from medicinal preparations.

5. Applied high performance liquid chromatography has been used to perform calibration curve from the resulting alprazolam, estazolam and triazolam qualitative evaluation, and therefor has shown that limits of detection of alprazolam is 0,01 µg/ml, estazolam 0, 012 µg/ml, triazolam 0, 020 µg/ml. Limit of quantification of alprazolam is 0, 022 µg/ml, estazolam 0, 025 µg/ml, triazolam 0, 045 µg/ml.
PRACTICAL RECOMMENDATIONS

Taking into consideration obtained results while performing thin layer chromatography and high performance liquid chromatography methodics can be concluded to be suitable for alprazolam, estazolam and triazolam qualitative assessment of medicinal preparations either separately or in mixture solutions. It is appropriate to develop extensive investigations in order to adapt the methodology selected by thin layer chromatography, a quantitative analysis.

In order to expand use of selected methods of toxicological analysis in practice it is appropriate to carry out detailed studies to evaluate the effectiveness of methods determining the investigative materials in pharmaceutical preparations.
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