The increasing drug consumption in Lithuania and all over the world makes us think about the negative consequences - the risk of toxicity. Fast and accurate identification of material that caused the poisoning reduces the probability in death cases and makes easier to determine the main cause of death. The results have shown that the most appropriate systems of solvents for qualitative analysis by TLC method of the mixture consisting of alprazolam, codeine and paracetamol are: system “D” (trichloromethane : acetone : conc. ammonia = 55 : 40 : 5 (v/v/v)) and system “F” (trichloromethane : diethyl ether : isobutanol : conc. ammonia = 50 : 30 : 15 : 5 (v/v/v/v)). For qualitative analysis of the mixture consisting of alprazolam, codeine and paracetamol by HPLC method the chromatographic column ACE C18 (25 cm × 4.6 mm × 5 µm), gradient elution mode (mixture of 3% acetic acid and methanol and the flow rate 1 mL/min have been used. The injection volume was 10 µL. Photodiode array detector (210 ñ 240 nm range) has been used. UV absorption spectra of materials measured using photodiode array detector have been identical to those presented in the scientific literature.

Keywords: alprazolam, codeine, paracetamol, TLC, HPLC.
medicinal products which may cause fatal poisonings when used in inappropriate doses. Moreover, the practice shows the trend of administration growth of following products, and thus the possibility of severe poisonings.

In order to optimize the work of toxicological laboratory in the case of determination of potential causes of poisoning, it is appropriate to use analytical methods which could help to identify a number of materials per analysis. Therefore, alprazolam, codeine and paracetamol can be used separately or in combination. For example; in the case of cold and depression, alprazolam and paracetamol and codeine is possible; in the case of cough - paracetamol and codeine. Because paracetamol and paracetamol/codeine containing medicinal preparations in Lithuania are sold without a prescription, the possibility of overdosage of these substances increases.

The aim of the work was to optimize the thin-layer chromatography and high performance liquid chromatography methods suitable for alprazolam, codeine and paracetamol mixture separation and qualitative analysis.

EXPERIMENTAL

Materials and Methods

Thin-layer chromatography (TLC) method

Chromatographic plates coated with sorption mass of silica gel were used for analysis (plates dimensions 20 x 20 cm, silica gel 60 F254, Merck, Germany). A glass chambers with ground glass lids were used for chromatography. The samples of analyzed substance were taken using glass capillaries with a volume of 10 µL. The eluent systems were prepared by mixing variable volumes of solvents: ethanol, trichloromethane, diethyl ether, conc. ammonia, acetone, isobutanol, isopropanol. Chromatographic plates (after drying) were visualized using Dragendorff reagent (modified by Munje) or UV light lamp (254 nm, 365 nm). Test solutions were prepared by dissolution of standards - alprazolam, codeine and paracetamol (Sigma-Aldrich, USA) - in methanol.

In that case the standard solutions of alprazolam (AE), paracetamol (PE) and codeine (KE) were prepared of concentration 0.1 mg/mL.

The mixture of components (APKE) was prepared by mixing of an equal parts (1 mL) of each standard solution.

High performance liquid chromatography (HPLC) method

For optimal determination conditions chromatograph Waters 2695 with a photodiode array detector (Waters 996, 210-400 nm wavelength range) was used. Separation of samples was performed using ACE C18 chromatographical column (25 cm x 4.6 mm) which sorbent particle size is 5 µm. Methanol, purified water (prepared by “Millipore” (USA) water purification system), 3% acetic acid aqueous solution, phosphate buffer (pH = 7), 0.1 % trifluoroacetic acid and acetonitrile were used for eluent systems preparation. Test solutions were prepared by dissolution of standards - alprazolam, codeine and paracetamol (Sigma-Aldrich, USA) - in methanol.

In that case the standard solutions of alprazolam (AE), paracetamol (PE) and codeine (KE) were prepared of concentration 0.1 mg/mL.

The mixture of components (APKE) was prepared by mixing of an equal parts (1 mL) of each standard solution.

RESULTS AND DISCUSSION

Optimization of TLC method

Selection of the visualization reagent

After the finishing of tests, in order to evaluate which visualizer is most suitable for all three substances identification, two methods of spots visualization have been checked: spraying with Dragendorff reagent (modified by Munje) and visualization using UV light (254 nm, 365 nm).

In both cases the spots of all substances were clearly visible on the chromatographic plate. Use of UV light in comparison with Dragendorff reagent is less expensive, easier to apply and leads to a lower operative personal contact with the active chemical reagents.

Selection of the eluent

Searching for the more acceptable eluent, the components of solvents and their ratio were changed in several solvent systems. Solvent system suitability was assessed according to standard alprazolam, codeine and paracetamol solutions drift spots on the chromatogram. By comparison of these values 6 solvent systems (in volumetric ratios) were selected as suitable for the identification of compounds in a mixture:

- system „A“ - trichloromethane : diethyl ether : isopropanol = 35 : 35 : 30;
- system „B“ - trichloromethane : diethyl ether : isobutanol = 35 : 35 : 30;
- system „C“ - trichloromethane : diethyl ether : conc. ammonia = 45 : 45 : 10;
Investigation of a mixture containing alprazolam, codeine, and...

- system „E“ - ethanol : acetone : conc. ammonia = 75 : 20 : 5;

Analysis of the solution of a mixture has been repeated using the different solvent systems five times. The statistical data evaluation has been performed by calculation of the arithmetic average \( X \) of the values of obtained \( R_f \) values, standard deviation SD, relative error RE at a confidence level of 0.95, confidence interval CI, when the error probability \( p < 0.05 \). (Table 1).

Table 1. Statistic evaluation of \( R_f \) values of the test substances (alprazolam, codeine, paracetamol) using solvent systems „A“, „B“, „C“, „D“, „E“ and „F“ in volumetric ratios.

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Test substance</th>
<th>( R_f ) average</th>
<th>Standard deviation (SD)</th>
<th>Relative error (RE)</th>
<th>Confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System „A“</td>
<td>alprazolam</td>
<td>0.58</td>
<td>0.008</td>
<td>0.004</td>
<td>0.58-0.59</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : isopropanol</td>
<td>codeine</td>
<td>0.17</td>
<td>0.005</td>
<td>0.002</td>
<td>0.16-0.17</td>
</tr>
<tr>
<td>35 : 35 : 30</td>
<td>paracetamol</td>
<td>0.59</td>
<td>0.002</td>
<td>0.002</td>
<td>0.58-0.58</td>
</tr>
<tr>
<td>System „B“</td>
<td>alprazolam</td>
<td>0.39</td>
<td>0.009</td>
<td>0.004</td>
<td>0.39-0.41</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : isobutanol</td>
<td>codeine</td>
<td>0.10</td>
<td>0.011</td>
<td>0.005</td>
<td>0.09-0.11</td>
</tr>
<tr>
<td>35 : 35 : 30</td>
<td>paracetamol</td>
<td>0.63</td>
<td>0.008</td>
<td>0.003</td>
<td>0.63-0.64</td>
</tr>
<tr>
<td>System „C“</td>
<td>alprazolam</td>
<td>0.92</td>
<td>0.008</td>
<td>0.004</td>
<td>0.91-0.93</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : conc. ammonia</td>
<td>codeine</td>
<td>0.92</td>
<td>0.005</td>
<td>0.002</td>
<td>0.91-0.92</td>
</tr>
<tr>
<td>45 : 45 : 10</td>
<td>paracetamol</td>
<td>0.78</td>
<td>0.007</td>
<td>0.003</td>
<td>0.78-0.79</td>
</tr>
<tr>
<td>System „D“</td>
<td>alprazolam</td>
<td>0.82</td>
<td>0.006</td>
<td>0.003</td>
<td>0.81-0.82</td>
</tr>
<tr>
<td>trichloromethane : acetone : conc. ammonia</td>
<td>codeine</td>
<td>0.69</td>
<td>0.007</td>
<td>0.003</td>
<td>0.6-0.80.70</td>
</tr>
<tr>
<td>55 : 40 : 5</td>
<td>paracetamol</td>
<td>0.49</td>
<td>0.008</td>
<td>0.004</td>
<td>0.4-0.80.50</td>
</tr>
<tr>
<td>System „E“</td>
<td>alprazolam</td>
<td>0.85</td>
<td>0.009</td>
<td>0.004</td>
<td>0.85-0.86</td>
</tr>
<tr>
<td>ethanol : acetone : conc. ammonia</td>
<td>codeine</td>
<td>0.72</td>
<td>0.0010</td>
<td>0.004</td>
<td>0.71-0.73</td>
</tr>
<tr>
<td>75 : 20 : 5</td>
<td>paracetamol</td>
<td>0.84</td>
<td>0.006</td>
<td>0.003</td>
<td>0.83-0.84</td>
</tr>
<tr>
<td>System „F“</td>
<td>alprazolam</td>
<td>0.86</td>
<td>0.004</td>
<td>0.002</td>
<td>0.85-0.86</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : isobutanol : conc. ammonia</td>
<td>codeine</td>
<td>0.75</td>
<td>0.008</td>
<td>0.004</td>
<td>0.74-0.75</td>
</tr>
<tr>
<td>50 : 30 : 15 : 5</td>
<td>paracetamol</td>
<td>0.45</td>
<td>0.007</td>
<td>0.003</td>
<td>0.44-0.45</td>
</tr>
</tbody>
</table>

Figure 1. The average \( R_f \) values of testing substances (alprazolam, codeine, paracetamol) in different systems of solvents
The repeatability error of used for duplicate tests solvent systems does not exceed the permissible limit of 0.05, so they all are suitable for alprazolam, codeine and paracetamol mixture qualitative analysis.

From Figure 1 data it follows that the average R\textsubscript{f} values of compounds in different solvent systems are not equivalent.

Application of solvent systems ÑAì and ÑBì shows the availability of analysis at a relatively low codeine R\textsubscript{f} values (R\textsubscript{f} < 0.17 and < 0.1, respectively), and its separation from the other components of the mixture is difficult. These two systems do not contain concentrated ammonia, which is present in other systems (ÑCì-ÑFî) and is important for efficient elution of codeine. Application of system ÑAì for a mixture analysis lead to alprazolam and paracetamol very close R\textsubscript{s} values (R\textsubscript{s} = 0.14), thus this solvent system is suitable for alprazolam or paracetamol separation from codeine (R\textsubscript{s} 5.85 and 6.0, respectively). ÑBì solvent system completely separates alprazolam and paracetamol (R\textsubscript{s} = 3.4).

Comparison of ÑAì and ÑBì solvent systems advantage shows that the elution of alprazolam is greater
Investigation of a mixture containing alprazolam, codeine, and... 617

with isopropanol than isobutanol (difference in \( R_f \) values > 0.19). For paracetamol elution difference between „A“ and „B“ systems is negligible (difference in \( R_f \) values < 0.4).

Using for analysis „C“ solvent system, codeine and alprazolam \( R_f \) values are close to each other \(( R_f = 0)\) and there is no possibility to separate these components of a mixture from each other. Concentrated ammonia presented in solvent system „C“ significantly increased codeine \( R_f \) value in comparison with „A“ and „B“ solvent systems. „C“ system eluent is suitable for codeine or alprazolam separation from paracetamol \(( R_f = 2.8)\).

Using for separation a mixture of substances „E“ solvent system, close \( R_f \) values for paracetamol and alprazolam could be calculated \(( R_f = 0.2)\), therefore this system is not appropriate for alprazolam separation from paracetamol. „E“ system can be used to separate alprazolam or paracetamol from codeine \(( R_f = 2.6 \) and 2.4, respectively).

The most suitable solvent systems for alprazolam, codeine and paracetamol mixture separation are systems „D“ and „F“ (see Figs. 2 and 3). Using „D“ system as an eluent the mixture components can be completely separated from each other: alprazolam – codeine \(( R_f = 3.25)\), alprazolam – paracetamol \(( R_f = 8.3)\) and codeine – paracetamol \(( R_f = 5.0)\). Similar results were obtained using as an eluent „F“ solvent system: alprazolam – codeine \(( R_f = 2.8)\), alprazolam – paracetamol \(( R_f = 10.25)\) and codeine -paracetamol \(( R_f = 7.5)\).

Optimization of HPLC method

For the mixture consisting of alprazolam, codeine and paracetamol analysis by high-performance liquid chromatography Waters 2695 chromato-

Figure 5. UV absorption spectra of paracetamol: A) literature data (data in two media: aqueous acid (a), aqueous alkali (b)) (8); B) obtained by measuring with photodiode array detector (aqueous acid)

Figure 6. UV absorption spectra of codeine: A) literature data (data in two media: aqueous acid (a), aqueous alkali (b)) [8]; B) obtained by measuring with photodiode array detector (aqueous acid)
graph equipped with the photodiode array detector (DAD) was used. Using this detector the mixture was analyzed according to retention time value and UV light absorption spectrum. The application of these two criteria for qualitative assessment results in high accuracy.

To optimize the HPLC method, which can be used for identification of alprazolam, codeine and paracetamol in the mixture, primarily, the assessment of the standard solutions in UV absorption spectra was performed. The obtained spectra for substances during analysis corresponded to the standard materials UV light absorption spectra provided in the literature database (8) - the following values of absorption maxima were determined: at 221 nm wavelength for alprazolam (see Fig. 4), at 245 nm wavelength for paracetamol (see Fig. 5) and at 285 nm wavelength for codeine (see Fig. 6).

**Chromatographic column selection**

In order to obtain the most suitable chromatographic column for the separation of the mixture of substances (APKE) several types of columns were used, but the best separation conditions were determined using ACE C18 (25 cm × 4 mm × 5 µm) chromatographic column.

**Eluent selection**

During the separation and identification analysis, the mixture of standard component solutions (APKE) was investigated using eluents of different composition. After the experiments using the condi-

![Chromatogram of optimal separation of codeine (1), paracetamol (2) and alprazolam (3) mixture](image)

**Table 2. Eluent composition (gradient mode).**

<table>
<thead>
<tr>
<th>Chromatographic time (min)</th>
<th>3% acetic acid solution (A) %</th>
<th>Methanol (B) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>00:00</td>
<td>70.0</td>
<td>30.0</td>
</tr>
<tr>
<td>28:00</td>
<td>20.0</td>
<td>80.0</td>
</tr>
<tr>
<td>29:00</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>31:00</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>32:00</td>
<td>70.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>

**Table 3. Statistic evaluation of retention time (Rt) values of the test substances (alprazolam, codeine, paracetamol).**

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Test substance</th>
<th>Retention time (Rt) average</th>
<th>Standard deviation (SD)</th>
<th>Relative error (RE)</th>
<th>Confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% acetic acid – methanol</td>
<td>alprazolam</td>
<td>29.81</td>
<td>0.009</td>
<td>0.004</td>
<td>29.81–29.83</td>
</tr>
<tr>
<td></td>
<td>codeine</td>
<td>6.78</td>
<td>0.008</td>
<td>0.003</td>
<td>6.77–6.79</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>7.92</td>
<td>0.008</td>
<td>0.003</td>
<td>7.91–7.93</td>
</tr>
</tbody>
</table>
The separation of APKE was performed using as eluent a solvent system composed of 3% acetic acid aqueous solution (A) and methanol (B). The quantitative composition of the eluent was changed using gradient mode (Table 2).

During the separation analysis, the mobile phase flow rate was: 0.5, 1.0 and 1.5 mL/min. The optimal separation of substances was achieved under conditions when the eluent flow rate was 1.0 mL/min. The total chromatographic analysis time was 32 min (see Table 2, Fig. 7).

Mixture of alprazolam, codeine and paracetamol standards was rechromatographed under the specified conditions five times. The obtained data were evaluated statistically by calculation of an arithmetic average $X$ of the retention time ($R_t$) values, standard deviation $SD$, relative error $RE$ at a confidence level of 0.95, confidence interval, when the error probability $p = 0.05$. (Table 3).

During the chromatographic separation process of APKE as first codeine is eluted from chromatographic column (retention time ($R_t$) average is 6.78 min). Paracetamol is eluted second after codeine (retention time ($R_t$) average is 7.92 min) and finally, the elution of alprazolam occurs (retention time ($R_t$) average is 29.81 min) (Table 3).

After performed repeatable separations, the obtained results do not exceed the error probability $p < 0.05$, so the method is suitable for the mixture analysis.

**Separation method validation**

The methods obtained for the mixture components separation and identification have been validated using specificity criteria according to ICH guidelines.

TLC: the mixture components have been identified according to standard solutions $R_t$ values (Table 1, Fig. 2, Fig. 3).

HPLC: the mixture of components have been identified according to standard solutions retention time ($R_t$) values (Table 3, Fig. 7) and UV spectra, recorded by photodiode array detector (DAD) and found in appropriate literature sources. (see Figs. 4-6).

**CONCLUSION**

Optimized thin-layer and high performance liquid chromatography methods are suitable for alprazolam, codeine and paracetamol mixture separation and qualitative analysis.

**REFERENCES**


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