TLC Applications on separation and quantification of fat-soluble vitamins

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Abstract

The history of vitamins, one of the most important chapters in the history of Biochemistry, had a profound impact on health and wellbeing, as well as on understanding the catalytic processes acting in the metabolism of living organisms. Since antiquity it has been known that there is a direct relation between illness and nourishment. A great number of methods have been developed to determine the content of fat-soluble vitamins in pharmaceutical products.

Though much less accurate than the other methods recommended in monographs, the thin-layer chromatography – TLC – finds its well deserved place among the analysis methods, when the relatively costly equipments involved by other methods are not available.

Keywords: fat-soluble vitamins, thin-layer chromatography, pharmaceuticals.

Introduction

TLC is the most used method, it is applied to separate the components of a sample mixture by using the whole range of fundamental processes in chromatography: adsorption, distribution, exclusion – diffusion and ion exchange. [1]

A great part of the fat-soluble vitamins present in pharmaceutical or natural products is accompanied by a significant number of kindred compounds. This explains why the chromatographic methods are frequently used in the analysis of these compounds. TLC is an ideal method for them, as it is a simple, selective and sensitive method. [2]

Results and discussion

Specialized literature mentions various methods for identifying and dosing of vitamin A, both from pharmaceutical products and from biologic fluids. The European Pharmacopoeia [3] mentions 2 methods for identifying of vitamin A in various pharmaceutical products by using TLC, namely:

- by using Silica Gel F_{254} plates; prepare a solution containing approximately 3.3 U.I. of vitamin A/µL in cyclohexane, stabilized with 1 g/L solution of butyl hydroxytoluene; the reference solution contains approximately 0.01 mg retinol/µL ester (3.3 U.I. from each
ester/µL) in cyclohexane, stabilized with 1 g/L solution of butyl hydroxytoluene; 3 µL of each solution is spotted on the plate;
- for synthetic oily vitamin A concentrates prepared from synthetic retinol ester, the identification is performed by means of thin layer chromatography (TLC) using Silica Gel F254 plates with cyclohexane solution, stabilized with 1 g/L solution of butyl hydroxytoluene; the ultraviolet examination is made at 254 nm.

For vitamin D, the European Pharmacopoeia indicates the thin layer chromatography (TLC) using Silica Gel G plates as identification method from various pharmaceutical products; prepare a solution containing 0.25 g substance in ethylene chloride; prepare reference solutions as well; spot 10 µL of each solution on the plate.

For vitamin E, the European Pharmacopoeia indicates the thin layer chromatography (TLC) using Silica Gel HF254R plates as identification method from various pharmaceutical products; prepare a solution containing 10 mg substance in cyclohexane; prepare reference solutions as well; spot 10 µL of each solution on the plate.

In thin layer chromatography, vitamins A, D and E, as well as their provitamins and esters are easily influenced by the environment conditions; the risk of their degradation, even destruction, by oxidation was enhanced by their long-term exposure to air on wide surfaces. These phenomena were minimized by working in darkness, under nitrogen flow, at 0°C. [4]

The absorption thin layer chromatography was highly used within studies on vitamins D, the most frequently used adsorbent being silica. This material has a high adsorption capacity and allows for fine and quick separations of important quantities of analytes, without plate overload. Janecke and Maass-Goebels [5] were the first to achieve separations through adsorption TLC of vitamins D mixtures. They succeeded in separating these vitamins from various sterols, from degradation products and from carotenes, using Silica Gel G plates and various mobile phases: hexane, hexane/ethyl acetate (90/10 v/v) and chloroform. As a consequence, the normal-phase absorption thin layer chromatography was used within separations of mixtures containing fat-soluble vitamins (Table 1) [4-9].

Table 1. Applications of normal-phase TLC in the separation of complex mixtures containing liposoluble vitamins

<table>
<thead>
<tr>
<th>Sample composition</th>
<th>Chromatographic systems:</th>
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<tbody>
<tr>
<td></td>
<td>Stationary phase</td>
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<td></td>
<td>Mobile phase</td>
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<tr>
<td>Lipid extracts containing cholesterol, vitamin A esters, α-tocopherol acetate and</td>
<td>Silica Gel</td>
</tr>
<tr>
<td>vitamin D [4]</td>
<td>Cyclohexane/diethyl ether (1/1 v/v)</td>
</tr>
<tr>
<td>Fat-soluble vitamins, carotenes, quinones and chlorophyll [5]</td>
<td>Silica Gel</td>
</tr>
<tr>
<td></td>
<td>- Cyclohexane/diethyl ether (1/1 v/v)</td>
</tr>
<tr>
<td></td>
<td>- Chloroform, hexane/ethyl acetate (9/1 v/v)</td>
</tr>
<tr>
<td></td>
<td>- Cyclohexane/chloroform (6/4 v/v)</td>
</tr>
<tr>
<td></td>
<td>- Benzene/methanol (98/2 v/v)</td>
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<tr>
<td></td>
<td>14 individual solvents</td>
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<td></td>
<td>Best separations were obtained with: benzene, toluene, xylol and carbon tetrachloride</td>
</tr>
<tr>
<td>Vitamins A, D and E [7]</td>
<td>Silica Gel</td>
</tr>
<tr>
<td></td>
<td>Hexane/ethyl-methyl-ketone/dibutyl</td>
</tr>
</tbody>
</table>

These first surveys are the basis of the implementation of this technique in the identification and measurement of vitamin D in more complex samples, especially in food matrices [10,11]. Adsorption TLC allowed equally for the study of safe isomerisations of vitamins D. It also allowed for checking the hypothesis issued especially on the mechanism of isomerisation from provitamin D to vitamin D [12], from previtamin D to vitamin D and reverse [4,13,14], and from tachisterol to isotachisterol [15-17] and for determining the kinetics of these conversions. This technique was used for the separation of vitamins D and of their hydroxylate metabolites [18]. In the United States it has been for several years one of the reference USP techniques for vitamins D\textsubscript{2} and D\textsubscript{3} identification tests [19]. It is also used to check the purity of vitamin D\textsubscript{3} marked with radioactive isotopes [20].

Molecules kindred with vitamins D groups (sterols, vitamin D esters, etc.) could be separated by the latter on Silica Gel impregnated with silver nitrate, the separation depending on the number of double bonds in molecules [21].

The TLC analysis was used for vitamin D identification on Silica Gel in Complex Calcium III tablets, using as mobile phase cyclohexane-ether-benzene (8:4:1), detection by spraying with sulphuric acid-acetic anhydride-10% ethanol (1:1:8) and heating to 110\textdegree C for 5 minutes [22].

The analysis of a mixture including fat-soluble vitamins such as D\textsubscript{1}, D\textsubscript{2} and tocopherols was performed by using as mobile phase acetone-concentrated acetic acid (3:2), detection being made with a solution in chloroform of stibium chlorinate (III) [23].

The TLC method was applied using Silica Gel, mobile phase hexane-ether (9:1) with traces of formic acid and quantification through densitometry at 350 nm for retinoic acid and at 280 nm for vitamin E [24].

HPTLC analysis of vitamin D\textsubscript{3} was performed by using Silica Gel with cu hexane (5 cm, without saturation chamber) and after intermediary drying with cyclohexane/diethyl ether 1:1 (7 cm, in saturated chamber with solvent); chromatography in darkness far from UV light source; quantification through densitometry with absorbance at 268 nm [25].

HPTLC analysis from the human plasma of plasma retinol, of α-tocopherol and of tocopheryl acetate on Silica Gel was performed using as mobile phase chloroform-cyclohexane (55:45), quantification through densitometry at 290 nm, detection limits 160 ng/mL for vitamin A and 1.2 µg/mL for vitamin E [26].

Analysis of 14 isolated substances, characterised by their spectral properties, degradation products resulted from β-carotene and retinyl acetate was performed using as mobile phase hexane/ether (9:1) [27].

For derivates of vitamin A, TLC was applied using Silica Gel RP-2 and Silica Gel impregnated with 10% paraffin in cyclohexane, mobile phase methanol/water (19:1) [28].
TLC analysis was applied for identification of vitamin E with Silica Gel plates, using as mobile phase petroleum ether (60-90°C)/ethyl acetate (95:5), quantification through densitometry at 280 nm [29].

13 stationary phase – mobile phase systems were investigated for separating and identifying vitamins A, D and E from pharmaceutical products (vitamin A+D2 soft gelatinous capsules, vitamin A solution, vitamin E soft gelatinous capsules and fish oil soft gelatinous capsules) [30].

The systems providing the best results were: stationary phase Silica Gel 60 F254 on plastic foil from Merck and hexane/ether (9:1, v:v) or benzene/chloroform (1:1, v:v) as mobile phase, which were photo documented in UV at 254 nm and for which we found values Rf of 0.463 for vitamin A acetate and 0.065 for D2, respectively 0.186 for D2 and 0.703 for α-tocopherol acetate.

Semi-quantitative analysis of vitamins, performed based on the blacking-out curves of the corresponding spot as spotted substance mass function led to a second degree multinomial function (correlation coefficient R = 1, p = 0.0159) for vitamin A acetate and to regression lines for vitamins D2 and E acetate (correlation coefficient R = 0.98994, p = 0.01006, respectively R = 0.98191, p = 0.01809).

The results obtained allow for easy implementation of TLC method in an analysis laboratory.

Conclusions

The TLC identification and semi-quantitative dosage methods are generally robust; they need a minimum of equipments and reactive, and can be easily adapted in small analysis laboratories.

The TLC methods are easy to apply, do not take long sample preparation time and do not lead to interferences in case of pharmaceutical products containing low numbers of vitamins, such as vitamin A+D2 soft gelatinous capsules, vitamin A solution and vitamin E soft gelatinous capsules; they are more difficult to apply and interpret when used on more complex mixtures, such as fish oil soft gelatinous capsules.

References

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   d. Ibid., 13, 265, 1967.