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Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining

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Abstract

Thin-layer chromatography (TLC) was used to screen for acetylcholinesterase inhibitors from Amaryllidaceae extracts. The TLC plate was developed and then stained using Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), to detect acetylcholinesterase activity. The advantages of this TLC assay method were that we could dereplicate the known inhibitor galanthamine, widely occurring in Amaryllidaceae, at an early stage of the isolation procedure. Moreover, there is no disturbance from sample dissolving solvents as in the microplate assay, and it is a very simple method. The detection limits were 10–200 ng for several known acetylcholinesterase inhibitors tested, and it is thus more sensitive than UV or Dragendorff's reagent detection. Also the minimal detectable amount for an acetylcholinesterase inhibitor tested was much less than that needed for the microplate assay. We screened 15 Amaryllidaceae extracts using this TLC method, and chose candidates for acetylcholinesterase inhibitor isolation.

Keywords: Amaryllidaceae; Bioactivity staining; Enzyme inhibitors; Dithiobis-(nitrobenzoic acid)

1. Introduction

An important approach to treat Alzheimer's disease (AD) is directed to the inhibition of acetylcholinesterase (AchE). Based on the cholinergic hypothesis, a defect in the cholinergic system is involved in AD [1]. Some AchE inhibitors like physostigmine or tacrine are known to have limitations such as short half-life or side-effects like hepatotoxicity [2]. Galanthamine, a long-acting, selective, reversible, and competitive AchE inhibitor, is considered to be more effective in the treatment of AD and to have fewer limitations [3]. Recently it has received its first approval for the treatment for AD in Austria. Some other alkylpyridinium polymers [4], dehydroevodiamine [5], and carbamate type AchE inhibitors [6] have been reported, but because of bioavailability problems and possible side-effects, there still is great interest in finding better AchE inhibitors.

To screen the almost inexhaustible sources of natural products, an effective and fast assay system is needed. Another problem often encountered when looking for inhibitors, is the isolation of a known active compound. To avoid the time consuming isolation of already known active compounds, a dereplication step — recognizing the already known

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active compounds — is required in the beginning of the isolation. We used silica gel thin-layer chromatography (TLC) to solve this problem. A qualitative method for AchE activity measurement on a TLC plate was described by Kiely et al. [7], which is based on the reliable and most widely used method according to Ellman et al. [8]. But they did not develop the TLC plate after loading samples, and it was just to assess the presence of inhibitory activity. We applied this TLC assay method with some adjustments, after developing the plate in an appropriate solvent system. TLC is a quite simple and also effective method to identify known compounds. For example, the AchE inhibitor, galanthamine, which is known to occur in Amaryllidaceae, can be detected in extracts by TLC. So, by applying the assay on the TLC plate after developing in an appropriate solvent, the presence of galanthamine and/or other active compounds can be detected at the same time.

Since Amaryllidaceae are known to contain a wide variety of biologically active alkaloids [9–16], we screened 15 Amaryllidaceae extracts for AchE inhibitory activity using the developed method, aiming at finding inhibitors other than galanthamine.

2. Materials and methods

2.1. Chemicals and equipment

2.1.1. Buffers

The following buffers were used. Buffer A: 50 m*M* Tris–HCl, pH 8; buffer B: 50 m*M* Tris–HCl, pH 8, containing 0.1% bovine serum albumin (BSA); buffer C: 50 m*M* Tris–HCl, pH 8, containing 0.1 *M* NaCl and 0.02 *M* $MgCl_2 \cdot 6H_2O$; and buffer D: 50 m*M* NaH_2PO_4–Na_2HPO_4, pH 7.6.

2.1.2. Enzyme

Acetylcholinesterase from electric eel (type VI-s, lyophilized powder, 292 U/mg solid, 394 U/mg protein) was purchased from Sigma (St. Louis, MO, USA). Lyophilized enzyme was dissolved in buffer A to make 1000 U/ml stock solution, and further diluted with buffer B to get 0.22 U/ml enzyme for the microplate assay, or diluted with buffer A to get 3 U/ml enzyme for the TLC assay.

2.1.3. Substrate

Acetylthiocholine iodide (ATCI) was purchased from Sigma (St. Louis, MO, USA). For the TLC assay 1 m*M* ATCI in buffer A was used, and for the microplate assay 15 m*M* in Millipore water was used.

2.1.4. Ellman's reagent

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) was purchased from Sigma (St. Louis, MO, USA). For the TLC assay, 1 m*M* in buffer A was used, and for the microplate assay 3 m*M* in buffer C was used.

2.1.5. Dragendorff's reagent

The Munier Macheboeuf modification [17,18] was used. Solution A was prepared by dissolving 0.85 g of bismuth subnitrate in 50 ml of 20% acetic acid. Solution B was prepared by dissolving 8 g of potassium iodide in 20 ml of H_2O. Stock solutions of mixture of A and B 1:1 were diluted with 2 vol. of acetic acid and 10 vol. of H_2O at the time of spraying.

2.1.6. Reference AchE inhibitors

Galanthamine hydrobromide, physostigmine (eserine) and carbachol were purchased from Sigma (St. Louis, MO, USA). Pancracine, lycorine and tazettine were gifts from Dr. Serap Whitmer.

2.1.7. TLC plate

DC-Alufolien, Silicagel 60 F254, 0.2-mm thickness were purchased from Merck (Darmstadt, Germany).

2.1.8. Microplate reader

Bio-Rad microplate reader model 3550 UV was used to measure the absorbance at 405 nm for the enzyme reaction in the microplate assay.

2.1.9. Amaryllidaceae and Agavaceae plants

All plants were a kind gift from J. Leenen (W.F. Leenen, Sassenheim, NL), from where cultivated plants are available for further studies. They were: 1, *Hymenocallis*×*festalis* 'Zwanenburg'; 2, *Chlidanthus fragrans* Herb.; 3, *Narcissus* 'Avalanche' (Tazetta); 4, *Nerine bowdenii*; 5, *Narcissus* 'Grand Soleil d'Or' (Tazetta); 6, *Zephyranthes candida* (Lindl.) Herb.; 7, *Crinum*×*powellii* Baker; 8,

Polianthes tuberosa L.; 9, *Amaryllis belladonna* L.; 10, *Eucharis amazonica* Linden ex Planch.; 11, ×*Hippeastrelia*; 12, *Habranthus robustus* Herb. ex Sweet; 13, *Rhodophiala bifida* (Herb.) Traub; 14, *Hymenocallis* 'Sulphur Queen'; and 15, *Sprekelia formosissima* (L.) Herb.

2.2. Procedures

2.2.1. Extraction of samples

Both methanol and toluene extracts were made from each species of 15 different Amaryllidaceae. First, freeze-dried and chopped samples were extracted at room temperature with 10 ml solvent per gram of dried samples for 1 day, and then extracted again with the same amount of fresh solvent for 1 week. Both extracts were combined and evaporated under reduced pressure.

2.2.2. TLC assay

Methanol and toluene extracts of the samples were dissolved in methanol to a concentration of 10 mg/ml. Then 2.5 μl of each sample was spotted on the silica gel TLC plate and developed in the solvent chloroform:methanol 8:2; 2.5 μl of 0.1 m*M* galanthamine solution in methanol was also spotted as a reference. After developing the TLC plate, enzyme inhibitory activities of the developed spots were detected by spraying the substrate, dye and enzyme based on Ellman's method [7]:

$$\text{Acetylthiocholine} + H_2O \xrightarrow{\text{AchE}} \text{acetate} + \text{thiocholine}$$

$$\text{Thiocholine} + \text{DTNB} \leftrightharpoons \text{5-thio-2-nitrobenzoate (yellow color)} + \text{2-nitrobenzoate-5-mercaptothiocholine} \quad \text{(Scheme 1)}$$

The plate was sprayed with DTNB/ATCI reagent (1 m*M* DTNB and 1 m*M* ATCI in buffer A) until the silica was saturated with the solvent, but not so much that the spots ran off. It was allowed to dry for 3–5 min and then 3 U/ml of enzyme solution was sprayed. A yellow background appeared, with white spots for inhibiting compounds becoming visible after ~5 min. These were observed and recorded within 15 min because they disappeared in 20–30 min. To measure the detection limit, 1.5 μl of various concentrations (5 m*M*–5 μ*M*) of galanthamine, physostigmine, pancracine, lycorine, tazettine and carbachol were spotted on the TLC plate and processed as above for AchE assay, or detected under UV and sprayed with Dragendorff's reagent to visualize alkaloids. The minimum concentration which could be recognized by eye was considered as the detection limit.

2.2.3. Microplate assay

AchE activity was measured using a 96-well microplate reader [19] based on Ellman's method

Table 1
Detection limits of the TLC assay for acetylcholinesterase, UV detection and Dragendorff's reagent for several acetylcholinesterase inhibitors on a silica gel TLC plate

Alkaloid	TLC assay	UV detection[a]	Dragendorff's reagent
Galanthamine, μg	0.01	0.6	0.2
Physostigmine, μg	0.01	0.05	0.5
Pancracine, μg	0.1	0.1	0.4[b]
Carbachol, μg	0.1	>0.8[c]	0.5
Lycorine, μg	0.2	0.2	0.4[b]
Tazettine, μg	>0.5[c]	0.2	0.5[b]

To measure the detection limits, 1.5 μl of each compound at a concentration of 0.005–5 m*M* in methanol was loaded for each spot on the TLC plate, and the minimum concentration which could be recognized as a white spot on the yellow background by eye was considered as the detection limit expressed in amount of sample (μg) on each spot.

[a] Spots were detected under UV lamp at 254 nm.

[b] Spots were not detected right away, but an orange color developed later.

[c] Spots were not detected at this level.

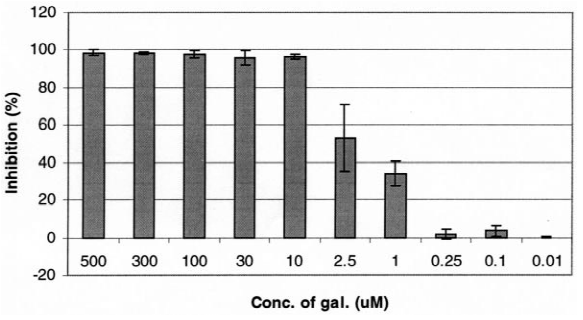

Fig. 1. Inhibition of acetylcholinesterase by galanthamine assayed on a microplate. A 25-μl amount of 15 m*M* ATCI, 125 μl of 3 m*M* DTNB, 50 μl of buffer, and 25 μl of galanthamine (5 m*M*–0.1 μ*M* in MeOH) were added in the 96-well plates, and the absorbance was measured at 405 nm. After 25 μl of 0.22 U/ml of enzyme was added, the absorbance was read again. Percentage of inhibition was calculated by comparing the reaction rates for the sample to the blank (MeOH). Error bars show the standard deviation of the measurements. The detection limit for galanthamine in the microplate assay was determined as final concentration of 1 μ*M* in the reaction mixture.

[7]. The enzyme hydrolyzes the substrate acetylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 405 nm. In the 96-well plates, 25 μl of 15 m*M* ATCI in water, 125 μl of 3 m*M* DTNB in buffer C, 50 μl of buffer B, 25 μl of sample (10 mg/ml in MeOH diluted ten times with buffer A, to give a concentration of 1 mg/ml) were added and the absorbance was measured at 405 nm every 13 s for five times. After 25 μl of 0.22 U/ml of enzyme was added, the absorbance was again read every 13 s for eight times. The rate of reactions were calculated by Microplate Manager software version 4.0 (Bio-Rad Laboratories). Any increase in absorbance due to the spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme from the rate after adding the enzyme. Percentage of inhibition was calculated by compar-

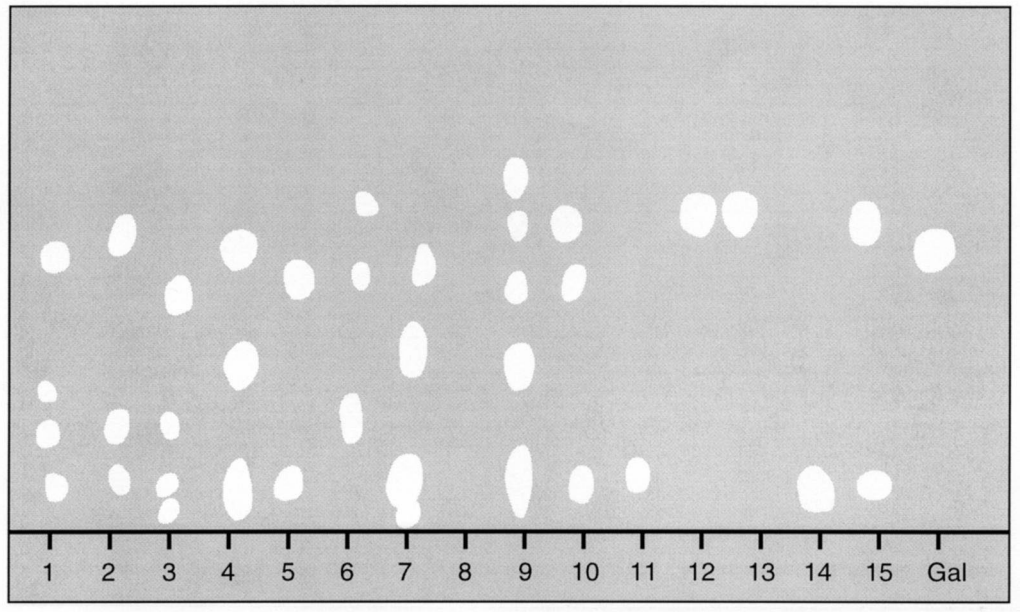

Fig. 2. Screening of some methanol extracts of Amaryllidaceae bulbs for acetylcholinesterase inhibitors using the TLC assay method. White spots on the yellow background represent the inhibition when 1 m*M* DTNB and 1 m*M* ATCI in 50 m*M* Tris–HCl, pH 8.0, was sprayed, followed by 3 U/ml of AchE spray. A total of 2.5 μl of 0.1 m*M* galanthamine or 10 mg/ml samples were loaded on the TLC plate, and developed in $CHCl_3$:MeOH 8:2. 1, *Hymenocallis*×*festalis* 'Zwanenburg'; 2, *C. fragrans* Herb.; 3, *Narcissus* 'Avalanche' (Tazetta); 4, *N. bowdenii*; 5, *Narcissus* 'Grand Soleil d'Or' (Tazetta); 6, *Z. candida* (Lindl.) Herb.; 7, *Crinum*×*powellii* Baker; 8, *P. tuberosa* L.; 9, *A. belladonna* L.; 10, *E. amazonica* Linden ex Planch.; 11, ×*Hippeastrelia*; 12, *H. robustus* Herb. ex Sweet; 13, *R. bifida* (Herb.) Traub; 14, *Hymenocallis* 'Sulphur Queen'; 15, *S. formosissima* (L.) Herb.

ing the rates for the sample to the blank (10% MeOH in buffer). To measure the detection limit, 5, 3, 1 m*M*, 300, 100, 25, 10, 2.5, 1, and 0.1 μ*M* of galanthamine in MeOH were used as samples and the assays were performed as above.

3. Results

3.1. Detection limits

The detection limits of the TLC assay for several known AchE inhibitors were in the range of 0.01–0.2 μg, depending on their activity (Table 1). This was much lower than the detection limit of UV absorption at 254 nm and Dragendorff's reagent for galanthamine and physostigmine. For example, the detection limits for both galanthamine and physostigmine were 0.01 μg with the TLC bioassay, whereas the limits of UV were 0.6 and 0.05 μg, and the limits for Dragendorff's reagent were 0.2 and 0.5 μg, respectively. Our method was also compared to the microplate assay for galanthamine. Inhibition of enzyme by galanthamine according to the microplate assay is shown in Fig. 1. The detection limit in final concentration of galanthamine in the reaction mixture of the microplate assay was 0.37 ng/μl (1 μ*M*), and that in TLC assay was 9.2 ng/μl (25 μ*M*). The amount of galanthamine for detection (mass sensitivity) was 92 ng (250 μl of 1 μ*M*) for one well in the microplate assay and 14 ng (1.5 μl of 25 μ*M*) for one spot in TLC assay. Therefore, in mass sensitivity, TLC assay is more sensitive than the microplate assay. From these results, our method is considered to be sensitive enough to be used as a qualitative assay method in screening for AchE inhibitors.

3.2. Screening of Amaryllidaceae for AchE inhibitors

The methanol and toluene extracts of 15 different Amaryllidaceae species along with galanthamine standard were developed on a TLC plate with the solvent system chloroform:methanol 8:2. The AchE inhibiting spots seen after spraying the substrate and

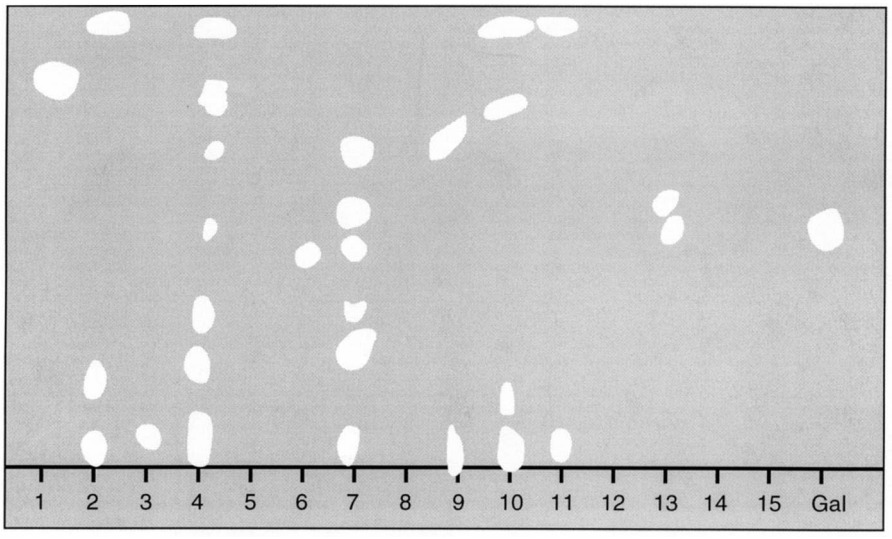

Fig. 3. Screening of some toluene extracts of Amaryllidaceae bulbs for acetylcholinesterase inhibitors using the TLC assay method. White spots on the yellow background represent the inhibition when 1 m*M* DTNB and 1 m*M* ATCI in 50 m*M* Tris–HCl, pH 8.0, was sprayed, followed by 3 U/ml of AchE spray. A total of 2.5 μl of 0.1 m*M* galanthamine or 10 mg/ml samples were loaded on the TLC plate, and developed in $CHCl_3$:MeOH 8:2. 1, *Hymenocallis*×*festalis* 'Zwanenburg'; 2, *C. fragrans* Herb.; 3, *Narcissus* 'Avalanche' (Tazetta); 4, *N. bowdenii*; 5, *Narcissus* 'Grand Soleil d'Or' (Tazetta); 6, *Z. candida* (Lindl.) Herb.; 7, *Crinum*×*powellii* Baker; 8, *P. tuberosa* L.; 9, *A. belladonna* L.; 10, *E. amazonica* Linden ex Planch.; 11, ×*Hippeastrelia*; 12, *H. robustus* Herb. ex Sweet; 13, *R. bifida* (Herb.) Traub; 14, *Hymenocallis* 'Sulphur Queen'; 15, *S. formosissima* (L.) Herb.

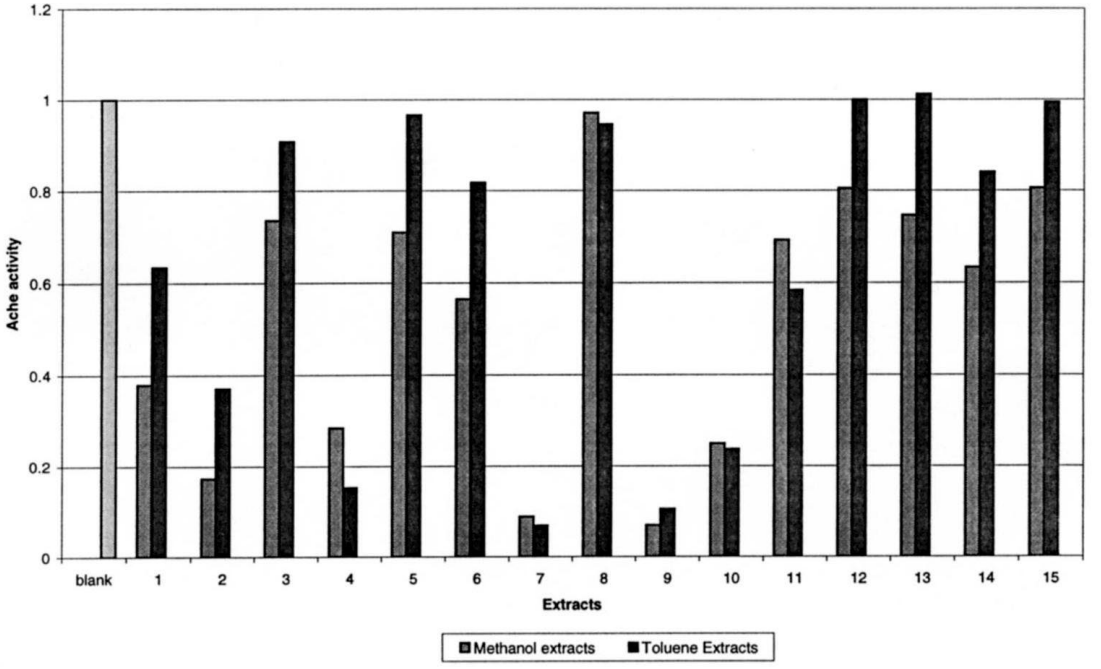

Fig. 4. Acetylcholinesterase inhibition by crude extracts of some Amaryllidaceae in the microplate assay. 1, *Hymenocallis*×*festalis* 'Zwanenburg'; 2, *C. fragrans* Herb.; 3, *Narcissus* 'Avalanche' (Tazetta); 4, *N. bowdenii*; 5, *Narcissus* 'Grand Soleil d'Or' (Tazetta); 6, *Z. candida* (Lindl.) Herb.; 7, *Crinum*×*powellii* Baker; 8, *P. tuberosa* L.; 9, *A. belladonna* L.; 10, *E. amazonica* Linden ex Planch.; 11, ×*Hippeastrelia*; 12, *H. robustus* Herb. ex Sweet; 13, *R. bifida* (Herb.) Traub; 14, *Hymenocallis* 'Sulphur Queen'; 15, *S. formosissima* (L.) Herb.

enzyme are shown in Figs. 2 and 3. AchE inhibitory activity of extracts measured by the microplate assay is shown in Fig. 4. *Crinum*×*powellii* Baker and *A. belladonna* L. showed strong inhibition in the microplate assay and also showed several inhibiting spots in the TLC assay which were not due to galanthamine. According to these results several candidates have been selected for further studies.

4. Discussion

In the TLC assay we determined the most suitable enzyme concentration as 3 U/ml. Even though the yellow background became more intense and white spots were more easily found when more concentrated enzyme was used, 3 U/ml gave acceptable results. Either Tris–HCl or NaH_2PO_4–Na_2HPO_4 buffer could be used for preparing the enzyme solution, DTNB and substrate, although DTNB is more easily soluble in phosphate buffer (buffer D). The amount of solution sprayed also has to be considered, because spraying too much liquid may lead to inspiring of the plate and broadening of the spots. Therefore, leaving it for ~5 min to let it dry between spraying the substrate and the enzyme is recommended. Several different types of TLC plates were compared for this assay. Silicagel plates (DC-Alufolien Silica gel 60 F254, 0.2-mm thickness; Silica gel 60 F254, glass, 0.25-mm thickness; Silica gel 60 F254, glass, 1-mm thickness), aluminium oxide plates (DC-Alufolien Al_2O_3 60 F254 neutral type E, and neutral type T, 0.2-mm thickness, Merck), reverse phase plates (DC-Alufolien RP-18 F254s, 0.2-mm thickness, Merck), cellulose plates (DC-Alufolien Cellulose F254, 0.1-mm thickness, Merck), polyamide plates (DC-Alufolien Polyamid 11 F254, 0.15-mm thickness, Merck, and Polygram Polyamid-6 UV254, 0.1-mm thickness, Macherey-Nagel, Duren, Germany) were compared, but only

silica and aluminium oxide neutral type E showed a yellow background and white inhibitory spots. Aluminium oxide type E gave a more apparent yellow background and white spots which lasted longer than on silica gel plates, and silica gel plates needed more enzyme to give a visible yellow color. But after the aluminium plates were developed in the TLC developing solvents, considerable tailing of the spots was observed, particularly clearly visible with the very sensitive bioactivity stainings. When samples were going to be checked for the AchE inhibitory activity without developing the TLC plate, alumina plates neutral Type E were better because these gave a more intense yellow color with less amount of enzyme. But in order to develop the TLC plates in the appropriate solvent before checking the inhibitory activity, silica gel plates had to be used to avoid the problem of tailing. Thickness of the silica gel plate also affected the results as 1-mm thickness plate could give a more apparent yellow background, but then more concentrated enzyme needed to be sprayed and more sample had to be loaded.

Even though the TLC assay is a qualitative method, we could recognize a known active compound (galanthamine) at an early stage and could thus choose the extracts which contained possible active compounds other than galanthamine. Another advantage of the TLC assay is that there is no disturbance of solvent which dissolves the sample before the assay. This is often a problem when the sample dissolves only in organic solvent which disturbs the assay. Using this TLC method together with the quantitative microplate assay, gives useful information for further purifying steps to dereplicate galanthamine and other known active compounds.

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