Improved Bioautographic Xanthine Oxidase Assay: Combining HPTLC separation and activity assessment for phytopharmaceutical research

Bräm S1, Danova K2, Meier B1, Wolfram E1

1 Zurich University of Applied Sciences, Life Sciences and Facility Management, Institute of Biotecnology, Wädenswil 8820, Switzerland
2 Institute of Organic Chemistry Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

Introduction

Xanthine oxidase (XO), a molybdenum containing flavoprotein, catalyses the oxidation of hypoxanthine and xanthine to uric acid under the formation of superoxide radicals and hydrogen peroxide. An overproduction of these free radicals in the human body is associated with diseases such as hyperuricemia, gout, hypertension, diabetes and different inflammatory diseases. Bioautography offers a rapid and simple tool for screening of secondary metabolites profiles of medicinal plants by HPTLC combined with screening of potential health benefits [1]. XO inhibitory effects can be directly visualised as white zones on a purple coloured thin layer chromatogram based on the reaction of superoxide radicals with nitroblue tetrazolium chloride (NBT). The aim of this work has been to optimize and validate a bioautographic XO inhibition assay first described by Ramallo et al. to obtain reliable and reproducible results [2]. The validated XO Assay has been tested using extracts of Camellia sinensis as known XO inhibitor as well as different ex situ and in situ extracts of Artemisia alba [3].

Validation aspects of the Bioautographic assay

**Bioautographic Assay**

- **XO-Activity**
  - Bioautographic XO-Assays were performed using Dry Extract (500µg) with 10x10 cm HPTLC plates. Incubation time and temperature were adjusted according to the AOX properties.

- **Buffer systems**
  - HPTLC Layer spotted with decreasing amounts of Allopurinol and Allopurinol standards.
  - Buffer systems with secondary ions such as Ca²⁺ showed to contain XO inhibitory effects. Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) interacted with the positive control Allopurinol. Most appropriate for the XO Assay was the use of potassium phosphate buffer at a pH of 7.6.

- **Inhibitory effects of solvent mixtures**
  - Bioautographic assay subsequently after plate development, plant extracts were spotted with Dihydroxyacetone, Alloxazine and Iodine. XO inhibition due to Allopurinol could be revealed down to an applied amount of 10µg dry weight (dw).

- **Buffer systems with secondary ions such as Ca²⁺**
  - Bioautographic assay was validated by including positive and negative control samples.

- **Bioautographic assay was performed using potassium phosphate buffer at a pH of 7.6.**

- **Solvent residues on HPTLC plate lead to a reduced plate staining when XO bioautographic assay was performed subsequently after plate development.** Therefore developed HPTLC plates should be fully dried for complete removal of solvents.

- **Since this assay uses superefficient activity for the screening of xanthine oxidase activity, superoxide radical scavengers can also generate positive results on the HPTLC plate.** However, such compounds can be differentiated from pure XO inhibitors by the direct measurement of uric acid by using a standard XO microtitreplate assay [4].

Results

**Bioautographic XO inhibition detection on spotted and developed HPTLC layers spotted with decreasing amounts of extracts and reference substances**

**HPTLC Layer spotted with decreasing amounts of Allopurinol and stained with the XO Assay**

**XO-Assay**

- **Microtiter plate assay**
  - IC₅₀ was determined in a microtiter plate assay at an Allopurinol concentration of (8.47 ± 5.89) µg·mL⁻¹.
  - The total amount of 227ng Allopurinol per well was required to screen 50% XO inhibition can also be detected by using the bioautographic XO assay.

- **XO-assay is therefore suitable for a rapid screening of compounds with strong XO inhibitory properties allowing a simple yes or no answer directly on the HPTLC plate.**

**Assay improvements**

- Reproducible bioautographic assay procedure
- XO Assay was validated by including positive and negative control
- XO Assay scale up from 5x5cm TLC layer upon 10x10cm HPTLC plates
- Amount of XO agar solution could be reduced
- Reduction of initial xanthine oxidase activity

Materials and Methods

- **Plant material**
  - Green tea was purchased from a pharmacy. A. alba in alu samples (1.1; 1.2; 1.3) were collected in different regions of Bulgaria. The samples were dried at 70°C and transferred to ZHAW for analysis. A. alba in vitro samples were grown on regular MS media.

- **Sample preparation**
  - Dried leaves of Camellia sinensis and dried plant material of A. alba were extracted using MeOH and incubated for 10 min in ultrasonic bath prior to 12h extraction time in the fridge under light exclusion conditions. Before filtration all samples were tempered and shortly agitated using Vortex. All samples were stored at 4°C in the dark prior to analysis. Positive control samples were prepared freshly by dissolving Allopurinol in Methanol. Negative control sample contained Methanol only.

- **HPTLC equipment and conditions**
  - Equipment: CAMAG Atr 4, ADC. TLC Visualizer and VisionCats Software. Stationary phase: HPTLC Silica Gel 60 F 254 aluminium sheets and glass plates 10x10 cm (Merk). Separation was achieved using solvent mixture 100:26:11:11 (v:v) Ethylacetate: Water: Acetic acid: Formic acid for a distance of 80 mm. Extract application volume was 10µL. Extracts and reference substances were prepared in Methanol. Reference application volume was 10µL.

- **XO Microtiter plate Assay**
  - XO Microtiter plate Assay XO Microtiter plate Assay was performed according to Havlikova et al. by measuring uric acid production [4]. Absorption at 295nm was measured by a Biotek Synergy Micro Plate Reader.

- **Bioautographic Assay Procedure**
  - Incubation time and temperature were adjusted according to the AOX properties.
  - The solution was cooled to 50°C and potassium phosphate buffer (150mM, pH=7.6) was added and mixed by a magnetic stirrer.
  - The buffered aqueous solution was given in a plastic tube containing 1.5mL reduced tetramethylchloride solution.
  - Microbial xanthine oxidase (3.14mL⁻¹) was added and carefully mixed by inversion.
  - Approximately 10µl of the solution was added to a 10x10cm HPTLC layer. The HPTLC plate was incubated at room temperature in the dark for 20 min.
  - The plate was then immersed in 3mL xanthine solution at 45°C for 60min in the dark.
  - The final concentration of the assay components were 7.5mM, 2.5mM, 2.5mM, 2.5mM, 2.5mM, 2.5mM, 2.5mM, 2.5mM, 2.5mM, 2.5mM.

- **Pinzgau oxidase**

**Conclusion and Outlook**

- The optimized bioautographic Xanthine Oxidase inhibition assay is a rapid and valid research tool for the assessment of active secondary metabolites from medicinal plants.
- Allopurinol spots could be detected down to an applied amount of 50ng. Extracts of C. sinensis and A. alba showed also to contain constituents with XO inhibitory activity, that could be visually detected down to an applied amount of 10µg dry weight (dw) for C. sinensis extract and 100µg dw for A. alba. Inhibition of XO due to Apigemon could be revealed down to an applied mass of 50mg.

- Buffer systems with secondary ions such as Ca²⁺ showed to contain XO inhibitory effects. Tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) interacted with the positive control Allopurinol. Most appropriate for the bioautographic XO assay was the use of potassium phosphate buffer at a pH of 7.6.

- Solvent residues on HPTLC plate lead to a reduced plate staining when XO bioautographic assay was performed subsequently after plate development. Therefore developed HPTLC plates should be fully dried for complete removal of solvents.

- Since this assay uses superefficient activity for the screening of xanthine oxidase activity, superoxide radical scavengers can also generate positive results on the HPTLC plate. However, such compounds can be differentiated from pure XO inhibitors by the direct measurement of uric acid by using a standard XO microtiterplate assay [4].

Acknowledgements

We thank Swiss National Fond (SNF) for funding of the Bulgarian Swiss Research Project Grant No. IZEBZ2_142898 and DOZ.1153 and the ZHAW Wädenswil for financial support.

References


Dr. Evelyn Wolfram, evelyn.wolfram@zhaw.ch

Zurich University of Applied Sciences, Institute of Biotechnology
Phytopharmaceutical research group, Wiedenswil, Switzerland

Sarah Brünn, brunn@zhaw.ch

Sofia, Bulgaria

Dr. Evelyn Wolfram, evelyn.wolfram@zhaw.ch

Dr. Katina Danova, katina.danova@zhaw.ch

Bulgarian Academy of Sciences, Institute of Organic Chemistry with Center of Phytochemistry, Sofia, Bulgaria