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Immunoassay for determination of trilobolide

Lukáš Huml [a,1], Michal Jurášek [a,1], Petra Mikšátková [a], Tomáš Zimmermann [a], Pavla Tomanová [a], Miloš Buděšínský [b], Zdeňka Rottnerová [a], Markéta Šimková [a], Juraj Harmatha [a,b], Eva Kmoníčková [c,d], Oldřich Lapčík [a,*], Pavel B. Drašar [a,*]

[a] University of Chemistry and Technology Prague, Faculty of Food and Biochemical Technology [342], CZ-166 28 Prague, Czech Republic
[b] Institute of Organic Chemistry and Biochemistry, CZ-166 10 Prague, Czech Republic
[c] Institute of Experimental Medicine, AS CR, v.v.i., CZ-142 20 Prague, Czech Republic
[d] Charles University in Prague, Faculty of Medicine and Biomedical Center in Pilsen, CZ-301 66 Pilsen, Czech Republic

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ABSTRACT

Trilobolide (Tb) is a pharmacologically interesting sesquiterpene lactone isolated from *Laser trilobum* (L.) Borkh. Structural relation to a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin bring promising prospects for Tb to be used in the development of new anti-cancer drugs. As long as there are still unanswered questions regarding its investigation, a need for novel analytical tools emerge. Since immunoassays serve as one of powerful tools within the investigation of natural products, the development of indirect competitive enzyme-linked immunosorbent assay (ELISA) utilizing coating based on avidin-biotin technology is described. In our set-up of ELISA, newly synthesized biotinylated Tb served as immobilized competitor. Tb-carboxymethyloxime-bovine serum albumin (BSA) and Tb-succinoyl-BSA conjugates were used separately for immunization of rabbits. Two sets of polyclonal antibodies (RAbs) were obtained. Antibodies against Tb-succinoyl-BSA conjugate (RAb No. 206) were chosen as the best. Under optimized conditions, limit of detection and 50% intercept of our ELISA were 849 pg/mL and 8.89 ng/mL, respectively. The cross-reactivity (CR) was tested on 10 structurally related compounds and CR did not exceed 6.1%. The reproducibility of the system is expressed as intra- and inter-assay coefficients of variation (9.7% and 11.4%, respectively). Based on conducted experiments, we proposed the use of ELISA for quantification of Tb in complex biological matrices such as plant extracts. A method was applied to analyze three extracts obtained from different parts of *L. trilobum*. Data obtained were compared to those acquired by UHPLC-MS/MS. The concordance between the methods (103–87%) showed the ability of ELISA to quantify Tb.

1. Introduction

Laser trilobum (L.) Borkh. (Apiaceae), known as horse caraway, Rosskümmel or Kefe cumin, is an aromatic herbaceous plant naturally occurring in several temperate regions of Europe and Asia. In the countries of the Near East, its seeds are mainly used as a spice [1,2]. During last decades, several sesquiterpene lactones (SLs) were isolated from this plant [3–5], however, only a few were found pharmacologically interesting. One of them is trilobolide (Tb, see Fig. 1), closely related SL to the extensively studied sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (Tg). Tb has attracted attention due to its closely linked pharmacological properties with Tg [6–11]. During research on Tb, tools such as fluorescent probes [12] and conventional chromatographic techniques [13] have been used for its determination. Thus far, however, no antibodies have been employed in such practice. The enzyme-linked immunosorbent assays (ELISA) are often relevant as highly specific, sensitive, cost-effective and user friendly tools within the investigation on natural products. Such methods might be convenient for numerous applications such as a tool for screening or even quantification of the analyte in complex biological matrices [14–18].

Abbreviations: BSA, bovine serum albumin; CMO, carboxymethyloxime; CR, cross-reactivity; ELISA, enzyme-linked immunosorbent assay; PEG, polyethylene glycol; RAbs, rabbit polyclonal antibodies; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SLs, sesquiterpene lactones; Tb, trilobolide; Tg, thapsigargin.

* Corresponding authors.
E-mail addresses: oldrich.lapcik@vscht.cz (O. Lapčík), drasarp@vscht.cz (P.B. Drašar).

[1] Contributed equally to this work.

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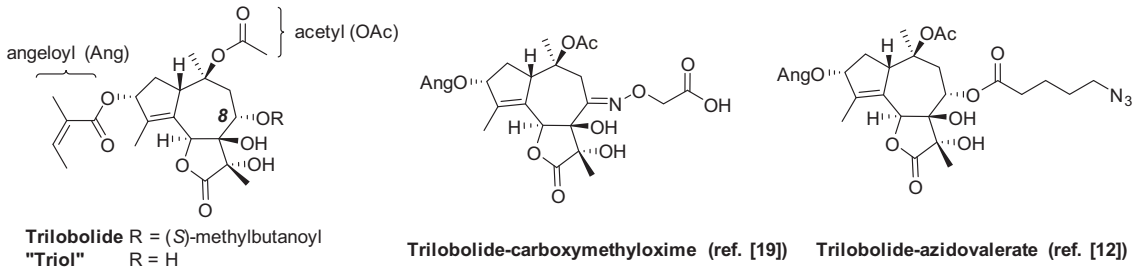

Fig. 1. Trilobolide and analogues used for this study.

For mentioned reasons, we designed and set up novel indirect competitive ELISA for the detection of Tb, in which avidin-biotin technology was used to immobilize antigen (see Fig. 2, part A). Such assay is characterized by the following steps: (a) avidin is coated on a microtiter plate; (b) biotinylated Tb is bound to biotin-binding sites on the avidin; (c) analyte and primary polyclonal antibody are applied; (d) peroxidase labelled secondary antibody followed by chromogenic substrate are added; (e) absorbance data are collected and evaluated.

In this study Tb-carboxymethyloxime [19] (Tb-CMO, see Fig. 1) and *N*-hydroxysuccinimide activated Tb-hemisuccinate **2** (see Scheme 1, part A) were conjugated to bovine serum albumin (BSA). Immunogens thus prepared were used separately for immunization whereby two sets of rabbit polyclonal antibodies (RAbs, two batches of antibodies for each immunogen) were obtained. Biotinylated component **3** (Scheme 1, part B,) was synthesized from previously described Tb-azidovalerate [12] (see Fig. 1) and acetylene-PEG_4-biotin via copper catalyzed click chemistry.

The ELISA was optimized and further focus was placed on its application on more complex biological matter. In this investigation, the ethanolic extracts of *L. trilobum* obtained from leaves, stems and seeds were used. Altogether, data from conducted experiments clearly demonstrated that our method can not only screen the presence of Tb, but when compared with UHPLC-MS/MS technique, we found it capable even for quantification of Tb in such a crude material.

2. Experimental

2.1. Chemicals and immunochemicals

3,3′,5,5′-Tetramethylbenzidine (TMB), dimethyl sulfoxide, hydrogen peroxide, gelatin from porcine skin, citrate-phosphate buffer tablets, Tween 20, bovine serum albumine (BSA), *N,N′*-dicyclohexylcarbodiimide (DCC), 4-*N*,*N*-dimethylaminopyridine (4-DMAP), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDCI), tris((1-benzyl-1*H*-1,2,3-triazolyl)-methyl)amine (TBTA), thapsigargin and avidin from egg white were purchased from Sigma (St. Louis, USA). Acetylene-PEG_4 biotin was supplied by Click Chemistry tools (Scottsdale, USA). The peroxidase labelled goat antibody against rabbit antibody (GAR/IgG(H + L)/PO) was purchased from Nordic-MUbio (Susteren, Netherlands). The microtiter plates Costar were supplied by Corning Incorporated (New York, USA). Standards of 2-acetoxytrilobolide, 2-deangeloylarchangelolide, acetyl-isomontanolide, archangelolide, eudeslaserolide, helenalin, laserolide, laserpitin, were from the laboratory collections (Juraj Harmatha). All other chemicals, reagents and solvents were used without further purification as purchased from commercial sources.

2.2. Synthetic and analytical general methods

The NMR spectra (^{1}H and ^{13}C) were recorded on Bruker Avance 600 instrument (Bruker, Billerica, USA) (^{1}H at 600 MHz; ^{13}C at 150.9 MHz) in $CDCl_3$ or CD_3OD. Homonuclear 2D-H,H-COSY and heteronuclear 2D-H,C-HSQC and 2D-H,C-HMBC experiments were used for structural assignment of hydrogen and carbon signals. Chemical shifts are given in δ (ppm). HRMS data were measured by Micro Q-TOF with ESI ionization (Thermo Scientific, Waltham, USA). FTIR spectra were measured on Nicolet iS10 model (Thermo Scientific, Waltham, USA) by ATR technique using KBr crystal (symbolism; *s* strong, *vs* very strong, *m* medium, *w* weak, *vw* very weak). Specific rotations were measured on Autopol VI polarimeter (Rudolph Research Analytical, Hackettstown, USA). For thin-layer chromatograms were used plates coated by silica gel bound with starch for detection in UV light (TLC Silica gel 60 F_{254}, Merck). For visualization diluted H_2SO_4 in MeOH was sprayed on TLC plates which were successively heated. For column chromatography silica gel (30–60 μm, SiliTech, MP Biomedicals) was used. For microwave synthesis Initiator Classic 355301 (Biotage, Uppsala, Sweden) was used.

2.3. Synthesis of immunogenic and biotinylated conjugates

2.3.1. Synthesis of Tb-8-O-succinate (*1*)

8-*O*-Demethylbutanoyl trilobolide was prepared according to previously descried method [12]. This derivative (100 mg, 0.23 mmol) was dissolved in CH_2Cl_2 (4 mL). Then succinic anhydride (39 mg, 0.39 mmol) and 4-DMAP (42 mg, 0.34 mmol) were added and the mixture was stirred for 20 h at RT. The solvent was evaporated under reduced pressure. The mixture was re-dissolved in EtOAc (30 mL) and washed with $KHSO_4$ (10% solution, 3 × 30 mL) and brine (1 × 30 mL). The organic layer was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The residue was applied on silicagel column (CH_2Cl_2/EtOAc 2/1→EtOAc). The product **1** (113 mg, 0.21 mmol) was isolated as slightly yellowish foamy solid in 92% yield. R_F = 0.55 in EtOAc + 1% HOAc. NMR data (see Supplementary, Table S1 and Fig. S6). HRMS-ESI: exact mass 538.20503 Da, found *m/z* 561.19438 $[M + Na]^+$ and 577.16858 $[M + K]^+$ (Supplementary, Fig. S1). IR: 3421 (*br*), 2928 (*m*), 2856 (*w*), 1785 (*m*), 1713 (*vs*), 1373 (*m*), 1244 (*vs*), 1158 (*vs*), 1042 (*m*), 1000 (*w*), 852 (*w*). $[\alpha]_D^{20} = -53.9$ (*c* = 1, MeOH/$CHCl_3$, 1/1).

2.3.2. Synthesis of NHS activated Tb-succinate hapten (*2*)

To a solution of hemisuccinate (**1**) and *N*-hydroxysuccinimide (70 mg, 0.61 mmol) in DMF (5 mL) was added EDCI (88 mg, 0.46 mmol) and the mixture was stirred for 20 h at RT. The solvent was removed under reduced pressure and the residue was chromatographed (hexanes/EtOAc, 2/1 → 1/2). The product **2** (104 mg, 0.16 mmol) was isolated as white foamy solid in 80% yield. R_F = 0.68 in EtOAc. NMR data (see Supplementary, Table S1 and