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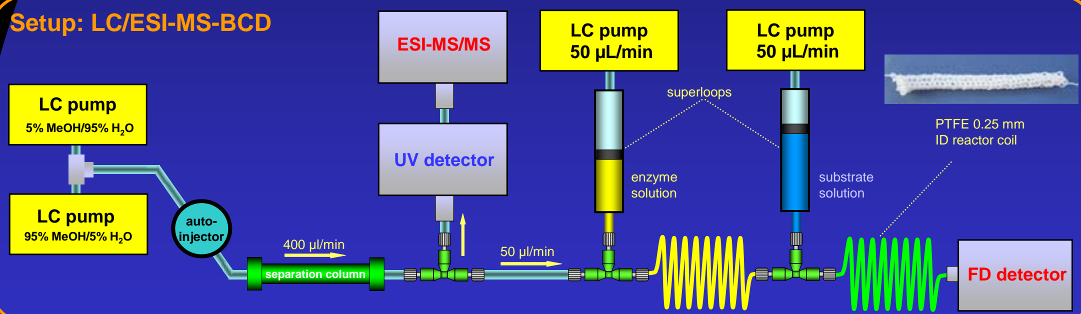
On-line detection of enzyme inhibitors using HPLC with biochemical detection

Background:

Enzymes play a key role in all biological processes. Therefore, the inhibition of enzymes by xenobiotics may lead to toxic effects. In contrast, inhibitors of certain enzymes, e.g. proteases in case of hypertension or thrombosis, are applied as drugs. The identification of these active compounds in natural samples, e.g. food constituents and plant extracts, is still a challenge in the life sciences. For this purpose we develop inhibitor screening methods based on the online-hyphenation of a continuous-flow enzyme assay serving as BCD to a reversed-phase (RP) separation. In contrast to common plate-reader techniques, this combination of separation and detection of biological activity allows the fast identification of active substances in complex mixtures. Here, we present the development of an LC-BCD method for serine protease inhibitors [1] and the application of a glutathione-S-transferase inhibitor screening method [2] to identify active patulin glutathione adducts.

Setup: LC/ESI-MS-BCD



Approach:

- Post-column addition of the enzyme of interest and the corresponding labeled substrate(s)
- Conversion of substrate to fluorescent product by continuously added enzyme in reactor coils → Steady state
- Inhibitory activity of eluting substances visible as decreased fluorescence → Online biochemical detection
- In parallel characterization of inhibitors by ESI-MS and MS/MS fragment spectra
- Validation and optimization of the BCD by flow-injection analysis (FIA) and the determination of IC_{50} values (Fig. 1)
- For reversed-phase gradient separations with a high content of organic modifier application of a counter gradient according to [1] (see poster M-LC-09)

Validation of the BCD signal

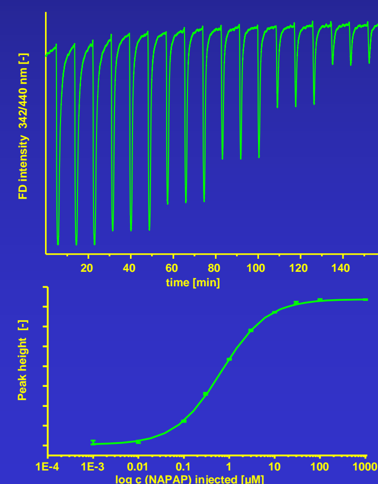


Fig. 1: Validation of a BCD for thrombin inhibition in flow-injection analysis (FIA) mode: (top): Triple injections (30, 10, 3, 1, 0.3, 0.1 µM) of a known inhibitor (NAPAP) and the corresponding IC_{50} curve (bottom).

→ Determined IC_{50} values for competitive inhibitors are comparable to classical plate-reader assays

LC-BCD for protease inhibitors

- Use of pure serine protease, e.g. thrombin and trypsin
- Application of a 4-aminomethylcoumarin (AMC)-labeled peptide (cyclohexylalanine-Ala-Arg-AMC) as substrate

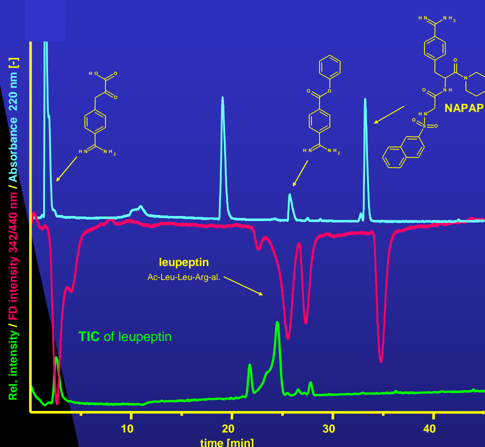


Fig. 2: Example: Gradient RP-separation of 6 different protease inhibitors and detection of their activity towards trypsin. The comparison of the UV trace (220 nm) and the fluorescence signal (342/420 nm) of the bioassay allows to distinguish between active and inactive substances. The structures of the active inhibitors are shown. The green chromatogramm shows the TIC of ESI(+)-MS analysis of a leupeptin, as a compound not visible in UV-detection.

The method provides:

- Stable BCD baseline (Fig. 2) during RP-separations applying a countergradient (poster M-LC-09)
- Parallel detection of different inhibitors
- Distinct correlation between peaks in BCD and UV/ESI

→ Suitable for the screening of complex mixtures

LC-BCD for glutathione-S-transferase (GST) inhibitors

- Use of rat liver cytosol as source of GST enzyme
- Substrate: Monochlorobimane – showing a strong fluorescence increase intensity after conjugation with glutathione (GSH)

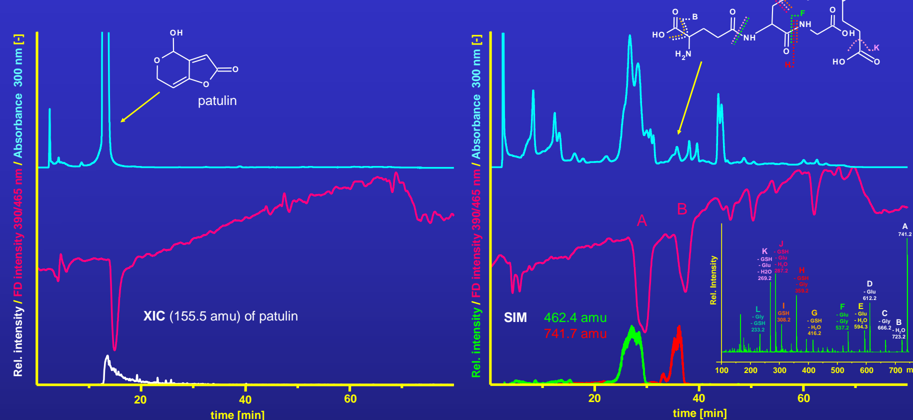


Fig. 3: Chromatogramms of a gradient RP-separation of patulin before (left) and after 24 h incubation with GSH (right). The comparison of the UV trace (300 nm) and the fluorescence signal (390/465 nm) of the bioassay allows to identify GST inhibitors. The chromatogramms below show the ESI(+)-MS of the [M+H]+ ions of patulin and two patulin-GSH adducts. In right corner the structure of a patulin-GSH adduct according to [3] with mass of the [M+H]+ ion of **peak B** is shown. The dotted lines indicate possible sites of fragmentation leading to the fragments, detected in the MS/MS spectrum (Insert).

Question: Are patulin and/or its GSH-adducts GST inhibitors ?

- Large signal in BCD for patulin (Fig. 3) → **Patulin itself is a GST inhibitor**
  Consistent plate reader results
- Patulin reacts with GSH giving rise to a complex mixture of patulin adducts (Fig. 3, UV-detector) At least 8 adducts were described by Fliege & Metzler [3] and were found in blood by Rychlik [4]
- Two formed adducts show a signal in BCD → **Patulin GSH-adducts are GST inhibitors**
- Active peaks show corresponding signals in ESI(+)-MS of [M+H]+ ions with the mass of two adducts described by Fliege & Metzler [3]. The observed MS/MS spectra of one adduct is in line with the suggested structure, too (Fig. 3).

Literature:

[1] N.H. Schebb et al. (2008) Anal Chem
[2] J. Kool et al. (2007) J Biomol Screen
[3] R. Fliege & M. Metzler (2000) Chem Res Toxicol
[4] M. Rychlik (2005) Nutrition

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