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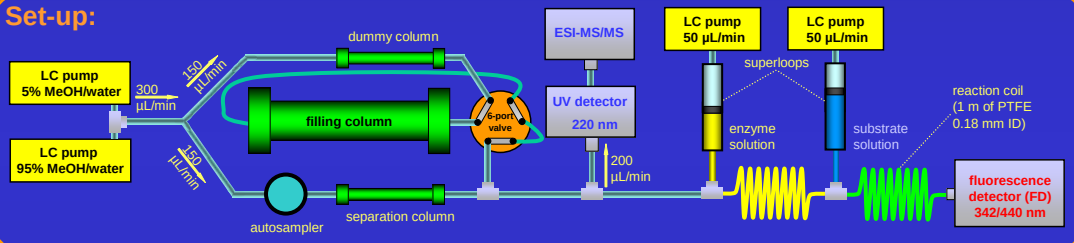
Screening for Protease Inhibitors Using LC/ESI-MS with a Continuous-Flow Enzyme Assay

Background:

Proteases play a key role in the regulation of physiological processes. Thus, specific inhibitors for these enzymes are potential drugs against diseases, e.g. thrombosis. The discovery of new inhibitors is still a challenge in pharmaceutical research. Therefore, we developed an online screening method for inhibitors of serine proteases. This method is based on a continuous-flow assay (CFA) for the determination of protease activity, which is hyphenated online to HPLC/ESI-MS system. In contrast to common plate-reader techniques, the combination of an HPLC/ESI-MS and detection of biological activity allows the fast identification of active substances even in complex mixtures.

Set-up:



Approach:

Continous- flow assay (CFA)

- Post-column addition of a serine protease and of 4-aminomethylcoumarin (AMC)-labelled peptide (cyclohexylalanine-Ala-Arg-AMC) as substrate.

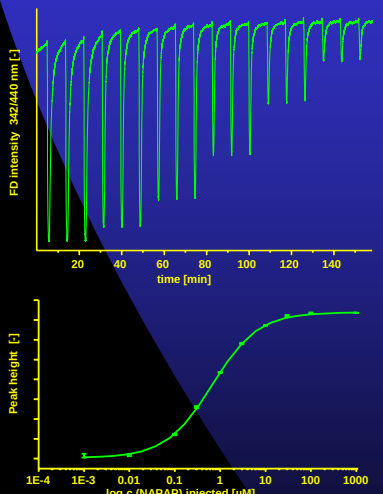


Fig. 1: Optimization / Validation of thrombin continuous-flow assay in FIA mode: (Top): Triple injections of known inhibitor NAPAP and the corresponding IC_{50} curve (bottom).

- Conversion of substrate by continuously added enzyme to free fluorescent AMC in reaction coils.
- Observation of inhibitory activity of eluting substances by decreased fluorescence intensity → negative peak.
- Validation and optimization of the CFA in flow-injection analyses (FIA) followed by the determination of IC_{50} values (Fig.1).

Countergradient system

- Applying a counter gradient to maintain a constant amount of MeOH in the CFA after gradient LC elution.
- Establishing the counter gradient based on a column coupling system incorporating a filling column to complete gradients.

LC/ESI-MS/CFA system

- Reversed phase gradient HPLC separation of mixtures and parallel detection by CFA and ESI-MS.
- By comparison of the activity peaks in CFA with those of ESI-MS, active inhibitors can be directly characterized by their mass or identified by their product ion spectra.

Results: Continuous-flow assay

- IC_{50} values of CFA were compared to plate-reader measurements:

→ CFA leads to similar IC_{50} values (Table 1).

Table 1: Comparison of IC_{50} values. The results of 3 known inhibitors of the developed continuous-flow assay (CFA) in FIA mode are compared to the results of a conventional fluorescence plate-reader assay.

	Thrombin	
Inhibitor	**IC_{50} (CFA) µM**	**IC_{50} (Plate) µM**
APP	3.2	5.2
APB	1.1	5.2
NAPAP	0.03	0.03
	Trypsin	
APP	1.5	1.4
APB	1.5	1.1
NAPAP	1	1.1

→ CFA allows to determine the inhibitory activity of eluting substances

Results: Countergradient system

- Stable countergradient (Fig. 3) requiring only one gradient pump.

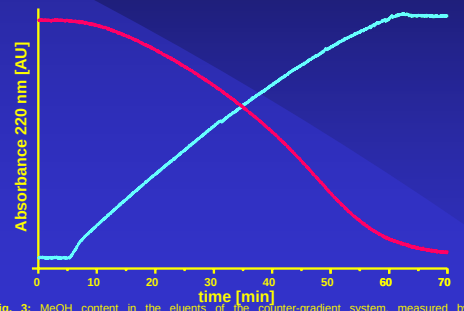


Fig. 3: MeOH content in the eluents of the counter-gradient system, measured by determination of eluent B (95% MeOH/water) containing 0.25% aceton and UV-detection at 220 nm: flow generated by the gradient pumps, counter gradient eluting from the filling column.

→ Isocratic conditions (+/-5 %) during gradient separation from 0-100 % organic solvent

Results: LC/ESI-MS-CFA system

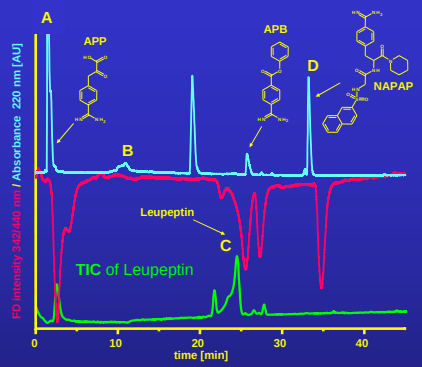


Fig. 4: Example: Gradient RP-separation of 6 different proteinase inhibitors and detection of their activity towards trypsin. The comparison of the UV trace (220 nm) and the fluorescence signal (342/420 nm) of the bioassay allows to distinguish between active and inactive substances. The structures of the active inhibitors are shown. The green chromatogramm shows the TIC of ESI(+)-MS analysis of a leupeptin standard.

MS/MS spectra

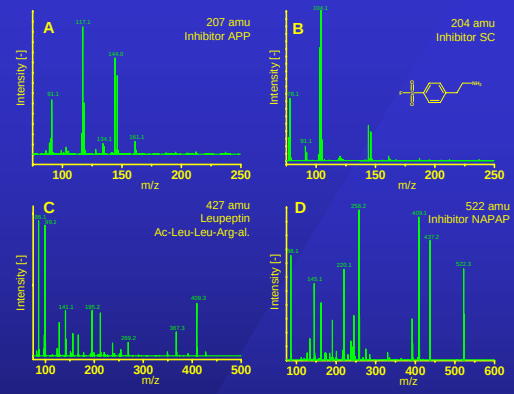


Fig. 5: ESI(+)-MS/MS fragment spectra of selected peaks of the analyis of mixture of inhibitors (Fig.4): **A** Inhibitor APP (m/z = 207), **B** irrevsible Inhibitor SC (m/z = 204) which shows no activity in CFA, **C** Main isoform of Leupeptin (m/z = 427) and **D** of the inhibitor NAPAP (m/z = 522 amu).

- Constant baseline of CFA with good peak shape and low peak broadening (Fig. 4).
- Distinct relation of signals of peaks in ESI(+)-MS and their activity in CFA (Fig-4-5).

→ allows direct identification of inhibitors by fragment spectra (Fig. 5).

Outlook:

- Application of the method to natural samples, e.g. snake venom.
- Miniaturization to reduce enzyme and substrate consumption.

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