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Determination of serine protease inhibitors by means of liquid chromatography and online biochemical detection

Background:

Proteases play a key role in the regulation of physiological processes. For example, angiotensin converting enzyme takes a center stage in regulation of blood pressure and thrombin and other serine proteases are responsible for blood coagulation. Thus, specific inhibitors for these enzymes are potential drugs against diseases such as hypertension and thrombosis. The search for new inhibitors is still a challenge in pharmaceutical research. For this purpose we developed an online screening method for inhibitors of serine proteases such as thrombin and trypsin: a continuous-flow assay (CFA) for determination of protease activity is hyphenated online to a reversed-phase (RP) separation. Decreased product formation measured in the CFA indicates the presence of an eluting inhibitor. 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.

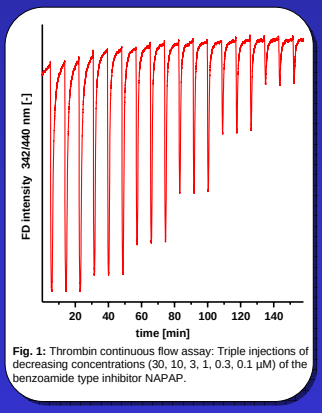


Fig. 1: Thrombin continuous flow assay: Triple injections of decreasing concentrations (30, 10, 3, 1, 0.3, 0.1 µM) of the benzoamide type inhibitor NAPAP.

Approach:

- Post-column addition of a serine protease and of 4-aminomethylcoumarin (AMC)-labelled peptide (cyclohexylalanine-Ala-Arg-AMC) as substrate.
- Conversion of substrate by continuously added enzyme to free fluorescent AMC in reactor coils (monitored by fluorescence detection (FD)).
- Direct observation of inhibitory activity of eluting substances by decreased fluorescence → online biochemical detection (BCD).
- Calibration and optimization of the BCD in flow-injection analyses (FIA) followed by the determination of IC_{50} values (Fig.1 and 2).
- Applying a counter gradient to maintain a constant amount of MeOH in the BCD in gradient HPLC mode.
- Creating the counter gradient with only one gradient pump system using a column coupling system incorporating a filling column to retain whole gradients.

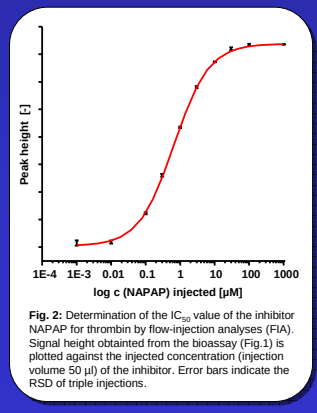
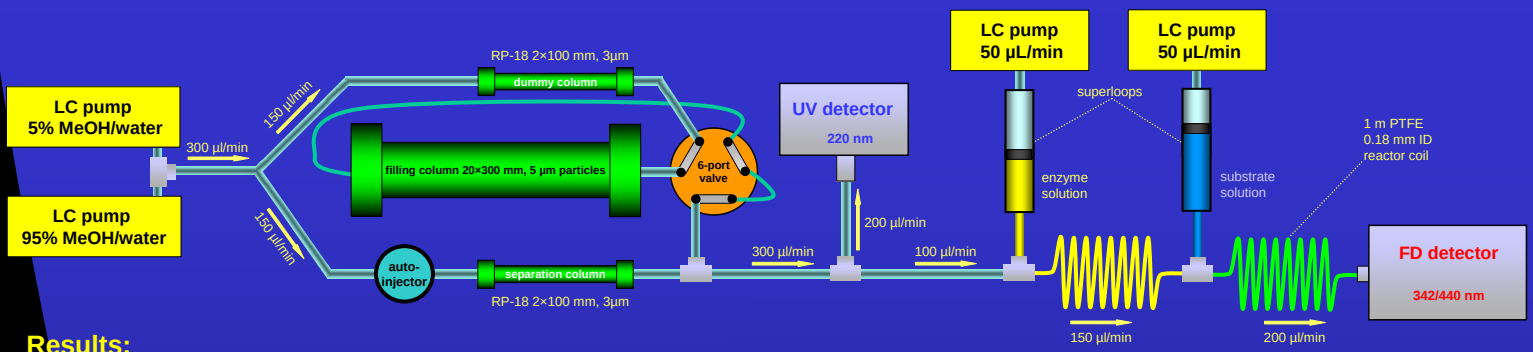


Fig. 2: Determination of the IC_{50} value of the inhibitor NAPAP for thrombin by flow-injection analyses (FIA). Signal height obtained from the bioassay (Fig.1) is plotted against the injected concentration (injection volume 50 µl) of the inhibitor. Error bars indicate the RSD of triple injections.



Results:

- Development of a stable and reproducible BCD for the measurement of inhibitory activity towards thrombin, chymotrypsin and trypsin (Fig. 1).
- Determined IC_{50} values by FIA (Fig. 2) are comparable to those measured by conventional plate reader assay (Table 1).
- Almost constant methanol content in BCD by a new rugged counter-gradient system requiring only one gradient pump (Fig. 3).
- Constant baseline of BCD with good peak shape and low peak broadening (Fig. 4).
- Distinct relation of signals of UV detection and their activity in BCD.

Table 1: IC_{50} values measured for 3 inhibitors (Fig.4) with the developed continuous-flow assay (CFA) compared to the results of a conventional fluorescence plate-reader assay. The injected concentration in the CFA was divided by the dilution factor of 20.4 regarding the dilution in the flow system.

Inhibitor	Trypsin		Thrombin	
	IC_{50} (CFA)	IC_{50} (Plate)	IC_{50} (CFA)	IC_{50} (Plate)
APP	1.3	1.4	3.2	3.6
APB	1.5	1.7	1.1	1.9
NAPAP	2	1.22	0.03	0.03

Acknowledgment:

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Example: Examination of a mixture of different known inhibitors and the resulting activity towards the digestion enzyme trypsin. RP-Separation: 5 min isocratic than 5-95% MeOH/water in 60 min with a counter gradient.

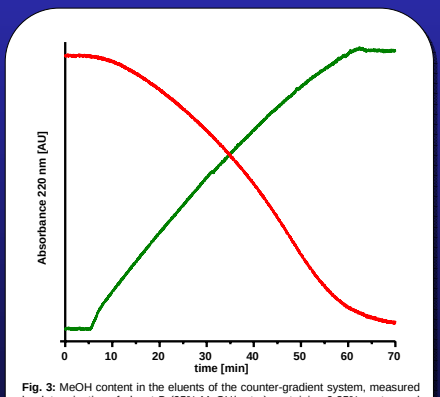

Fig. 3: MeOH content in the eluents of the counter-gradient system, measured by determination of eluent B (95% MeOH/water) containing 0.25% aceton and UV-detection at 220 nm: flow generated by the gradient pumps, counter gradient eluting from the filling column.

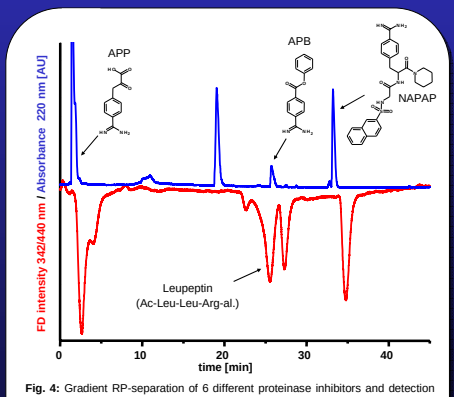

Fig. 4: Gradient RP-separation of 6 different proteinase inhibitors and detection of their acitivy towards trypsin. The comparison of the UV trace (220 nm) and the fluorescence signal (342/420 nm) of the bioassay allows to distinguish between active and inactive substances. The structures of the active inhibitors are shown.

Outlook:

- Application of the method to natural samples, e.g. snake venom.
- Hyphenation to ESI-MS in order to identify new inhibitors.
- Miniaturization to reduce enzyme and substrate consumption.