On-line biochemical detection of serine protease inhibitors hyphenated to liquid chromatography with a novel counter gradient system

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Enzymatic conversions play a crucial role in the regulation of all processes of life. In particular, proteases like thrombin, coagulation factors or angiotensin converting enzyme play a major role in the regulation of blood coagulation and blood pressure and are important targets for the development of drugs and diagnostic applications. Hence, it is a goal of pharmaceutical research to screen for new specific inhibitors, which can be used as drugs. Natural mixtures as extracts from plants and bacterial suspensions or snake venom are promising sources for new inhibitors. To identify inhibitors in these complex mixtures a fast and robust screening system containing a separation step is needed.

Therefore we are developing an online liquid chromatography-biochemical detection (LC-BCD) method for the separation and detection of protease inhibitors. The potential inhibitors are separated by RP-chromatography. A constant flow of a coumarin-labelled peptide as substrate and proteinase solution is added to the eluate by two additional HPLC pumps, applying superloops. In a reaction coil the highly fluorescent 7-Amino-4-methylcoumarin is released from the substrate by the protease, which is detected by a fluorescence detector. If an inhibitory compound is injected, less product is formed, observable as negative peak in the detected signal.

For RP gradient separations, a novel countergradient system was developed to guarantee constant solvent conditions in the BCD. The countergradient system is based on the retaining of complete gradients in an additional HPLC column with large void volume. I contrast to all other techniques to set up a countergradient, no additional LC pumps are needed. The developed counter gradient system was systematically evaluated applying different gradient programs. Hereby, we demonstrate that the concentration of solvent B remains constant (+- 7 %), when applying a gradient from 0-100% solvent B

The BCD was optimized in flow injection analyses (FIA) mode, without chromatographic separation. Finally the complete HPLC-BCD system was used to screen a small library of known inhibitors for thrombine and the mammalian digestion enzymes trypsin and chymotrypsin. The determined IC_{50} values were in good accordance to those of convential plate reader assays.