Automated precolumn derivatization procedures in HPLC for biomedical and clinical applications

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This thesis describes three automated precolumn derivatization procedures for the analysis of carboxylic group-containing compounds. After derivatization with a suitable label, the derivatives are separated on reversed-phase HPLC and detected by fluorescence.

In the analysis of organic compounds chromatographic techniques play an predominant role; for the analysis of polar compounds High Performance Liquid Chromatography, HPLC, is the method of choice. A major disadvantage of the technique is the lack of sensitive and selective detectors. Derivatization, chemical and/or physical alteration of the analyte, is often used to enhance sensitivity or selectivity. In Chapter 1 the pros and cons of derivatization are given. Next the automation of such reactions is discussed.

Chapter 2 deals with use of 4-bromomethyl-7-methoxycoumarin as labelling agent. In Section 2.1 a review of the applications of this compound and its analogues as derivatization agents is given. The next two sections discuss the optimization of the reaction and its automation. In Section 2.2 the role of the various constituents of the reaction mixture is reported along with the optimization of the HPLC separation; analytical data such as repeatability, detection limits and linearity are presented. The last section deals with the application of the method to the determination of the anti-epilectical drug valproic acid in human serum and in rat blood and brain dialysates. The present procedure for human serum is compared with an immuno-assay method routinely used in clinical laboratories. The animal studies were carried out to investigate the use of valproic acid as a model compound in pharmacological studies on blood-brain passage. Valproate could be determined in both blood and dialysates and the automated method has recently been used for the determination of the cellular and extra-cellular concentration of valproic acid in freely moving rats [50]. This study is not included in this thesis.

An important characteristic of 4-bromomethyl-7-methoxycoumarin is that it is only reactive in aprotic, i.e. non-polar, solvents. For that reason two highly interesting polar neuroactive compounds, N-acetylaspartate and N-acetylaspartylglutamate, can not be determined by means of procedures involving this label. For these analytes we developed a procedure using 2-aminoanthracene as label and carbodiimide as coupling agent; this work is presented in Chapter 3. The reaction is fast and requires only minimal sample pretreatment. N-Acetylaspartate and N-acetylaspartylglutamate levels in several brain regions and spinal cord were similar to those so far reported.

Attempts to investigate whether the present procedure is more sensitive than the well known reaction with o-phthaldialdehyde for the determination of amino acids was not successful; the amino acids did not react with 2-aminoanthracene. An

increase in sensitivity was, however, found using naphthalene-2,3-dicarboxaldehyde as label. In Chapter 4 relevant analytical data are given on the automated procedure for the determination of several amino group-containing compounds such as amino acids, small peptides, brain amines and some drugs.

The present thesis shows that commercial equipment and reagents are available which allow the set-up of fully automated on-line precolumn derivatization in combination with HPLC and fluorescence detection. We believe that the approach of automated on-line precolumn derivatization HPLC analysis carried out with, mainly, commercially available equipment will become an attractive alternative to off-line precolumn as well as on-line postcolumn procedures in many fields of research. Although the examples in the present work all deal with (bio)medical applications, the procedures can be easily be modified to be effective in areas such as environmental, agricultural or pharmaceutical analysis.