Analytical methods based on natural and artificial antibodies for determination of antiepileptic drugs

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thesis

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Introduction

During my doctoral work I have developed methods based on molecular recognition for the determination of antiepileptic drugs. In the first part of my work I have used natural antibodies for this purpose, while in the second part I have prepared artificial antibodies capable of molecular recognition via polymerisation. Due to the fact that epilepsy is one of the most frequent disease of the nervous system, 0,5% of the population is affected[1], the treatment is very important and has to be planned regularly. To avoid toxicity and ensure efficacy a continuous therapeutic monitoring is needed.

Immunoanalytical measurements

A novel type of flow injection immunoassay with fluorimetric detection was developed for the determination of phenytoin in serum. The competitive immunoreaction takes place in solution in a reaction coil of the flow injection system. The antibody bound phenytoin and free phenytoin are separated by size exclusion chromatography due to the large size difference and the fluorescent tags are detected in a fluorescent detector. During immunoassay method development optimisation of the chromatographic parameters that affected the separation (gel filtration media, mobile phase, column length, flow rate, incubation time) were identified and optimal values were determined. The analytical performance of the method was proved by validation. Applicability of the method for the determination of phenytoin in serum in clinical analysis was tested by comparison with an established reference method.

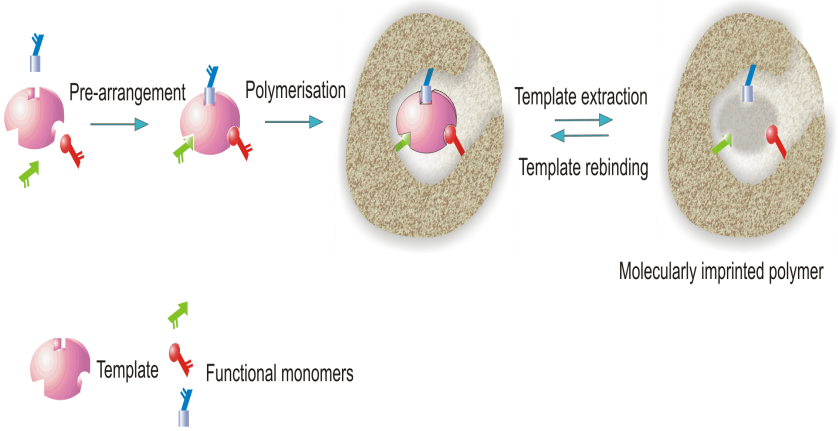
To demonstrate that the method developed is suitable and easily adaptable for the determination of drugs having small molecular weight, we proved the applicability of the method for the determination of phenobarbital in serum. After minimal optimisation of the parameters that affected the separation (incubation time) the method was validated for determination of phenobarbital is serum. The results obtained with our method show a good correlation with those of other methods already established in the clinical laboratory.

Our method is simple, cost-effective, relatively rapid and no high instrumentation is required. These advantages make this technique a viable candidate in hospital laboratories for the rapid determination of small (drug) molecules.

Molecularly imprinted polymers

In the second part of my docoral work I have synthetized and studied molecularly imprinted polymers. The polymers work as antibodies based on molecular recognition and in many cases exhibit antibody like affinity and selectivity for the template.

The molecularly imprinted polymer preparation (the non covalent imprinting, see Figure) involves three major steps. The first step is based on a *pre-arrangement* between the *template* molecule (phenytoin) and the *functional monomer*. The second step is the *polymerisation* procedure, which is followed by the *extraction* of the template from the polymer matrix. Template removal leaves cavities complementary in shape, size and functionality. In given conditions these cavities are able to selectively rebind the template from a complex mixture due to the fact that the imprints are complementary, sterically and chemically to the templating ligand.



Following optimisation of the polimerisation conditions (functional monomer, porogen) the molecularly imprinted polymer was prepared using methacrylamide as functional monomer. The results indicated that the polymer exhibited highly selective affinity for phenytoin. The selectivity of the imprinted polymer was investigated using phenytoin and it's metabolite, several structurally related compounds and compounds having similar lipophilities to phenytoin. Strengths and density of the specific bindig sites was calculated. Study of the recognition properties of the imprinted polymer in different solvent media was carried out and the role of water in the recognition

process was investigated. The results indicate that careful choice of the the solvents can disrupt non-specific binding, allowing a specific extraction of the template. A procedure for phenytoin determination from plasma samples using the molecularly imprinted polymer as solid-phase extraction sorbent was elaborated. The applicability of the procedure was proved by validation of the method. This was the first validated analytical procedure using molecularly imprinted polymers.

THESIS

1. I have developed a novel flow injection immunoassay based on gel chromatographic separation for the determination of phenytoin in serum. I have proved the analytical performance of the method and verified against an independent clinical method (^{125}I RIA RK 41 radioimmunoassay).
2. I have proved that after minimal optimisation the method is suitable for the determination of small (<1000Da) drug molecules. This was demonstrated via elaboration of a validated method for the determination of phenobarbital in serum.
3. I have synthetised for the first time molecularly imprinted polymer capable for the selective enrichment of phenytoin.
4. I have proved that two classes of binding sites are present in the imprinted polymer - high affinity binding sites with a binding constant of about 5675 M^{-1} and low affinity binding sites with a binding constant of about 257,8 M^{-1}.
5. Based on the selectivity results of the imprinted polymer using phenytoin, several structurally related compounds and compounds having similar lipophilicities to phenytoin I have shown that selectivity is based on the specific structrure of the phenytoin, not on the differences in the hydrophobicity. The hydantoin ring and the geometry of the phenytoin molecule are the principal factors that determine the selectivity.
6. Study of the recognition properties of the imprinted polymer in different solvent media was carried out. Based on these results I have elaborated a procedure for phenytoin determination from plasma samples using the molecularly imprinted polymer as solid-phase extraction sorbent. This was the first validated analytical procedure using molecularly imprinted polymers.

Publications related to the doctoral thesis:

1. **Andrea Bereczki**, Viola Horváth: "Novel type of flow injection immunoassay for determination of phenytoin from serum" *Anal. Chim. Acta*, **1999**, 391, 9-17.
2. **Andrea Bereczki**, Viola Horváth, Gerorge Horvai: "Immunoassay based determination of phenobarbital using size exclusion chromatography", *J. Chromatogr. B*, **2000**, 749, 215-223.
3. F. Lanza, A.J. Hall, B. Sellergren, **A. Bereczki**, G. Horvai, S. Bayoudh, P.A.G. Cormack, D. Sherrington: "Development of a semiautomated procedure for the synthesis and evaluation of molecularly imprinted polymers applied to the search for functional monomers for phenytoin and nifedipine", *Anal. Chim. Acta*, **2001**, 435, 91-106.
4. **Andrea Bereczki**, Antal Tolokán, George Horvai, Viola Horváth, Francesca Lanza, Andrew John Hall, Borje Sellergren: "Determination of phenytoin by molecularly imprinted solid phase extraction", *J. Chromatogr. A*, **2001**, 930, 31-38.

Other publication:

1. Róbert E. Gyurcsányi, **Andrea Bereczki**, Géza Nagy, Michael R. Neuman, Ernő Lindner: "Amperometric microcells for alkaline phosphatase assay", *Analyst*, **2002**, 127, 235-240.