DEVELOPING HIGH-SPEED HPLC SEPARATIONS FOR THE ANALYSIS OF BIOACTIVE COMPOUNDS

Boros Borbála

PhD thesis

University of Pécs
Faculty of Medicine
Hungary

Department of Biochemistry and Medical Chemistry

Pécs, 2007.

INTRODUCTION

These days, High-Performance Liquid Chromatography (HPLC) is one of the most important instrumental analytical methods. This statement should not be surprising if we take into account the almost unlimited opportunities offered by the principles and the applications of this method, as well as the enormous quantity of information we can come into possession through such measurements. This method meets the most important requirements existing vis-à-vis a modern analytical method, namely: the high speed and the high sensitivity of the analysis.

Like most instrumental analytical techniques, HPLC also spreads widely as a test procedure used by research and development laboratories. The unbroken trend of its prevalence is the result of the fact that the applications of fast and sensitive chromatographic procedures is gaining in popularity in routine testing, and that the industrial sector has quickly accepted and implemented this methodology. In the pharmaceutical industry, the role of HPLC methods has been and is on the increase in the testing and qualification of raw materials, intermediers, reaction mixtures, drug base materials, and ready-packed drug products.
The regulations and recommendations of various government agencies (FDA, OGYI) and international organisations (WHO, FOA) have significantly contributed to the popularity of this technique.

High Performance Liquid Chromatography has reached a very high technical level by these days and, it can be seen, that almost in any case, solving a new analytical problem comes hand in hand with research in chromatography to a certain level. There is not a single test scheme or procedure that would be suitable for all analytical problems that occur.

Parallel with the improvement of chromatographic equipment, it was also necessary to further develop the stationary phases (the special charges) as well as the columns used in the procedure.
Stationary phases are the very core of chromatographic separations, therefore development is the most intensive, also today, in the field of the improvement of charges.

When I develop a modern -sensitive and a few minutes long- separation technique for bioactive compounds, great care must be taken in selecting the proper chromatographic charge type. In liquid chromatographic separation procedures, *non-porous*, *micropellicular*, reverse phases charges with particle diameters of less than 3 μm are more and more often in use. Initially such charges were widely used in applications for fast analyses of biopolimers, and later they started to play a role also in the separation of organic compounds of low molecular weight.

OBJECTIVES SET FOR THE RESEARCH PROGRAMME

The objective of my investigation was to develop high-speed HPLC analytical methods for the separation of bioactive compounds of low and of high molecule weight, through the application of *non-porous*, micropellicular, reverse phases.

The goal was to select groups of compounds for the chromatographic analysis that had high significance in life sciences, in medical biological researches and in clinical analyses. The selected sample compounds are important from the aspect of practicality, and also to verify the novel properties of *non-porous*, micropellicular, reverse phases through compound groups that represent various separation problems (strong polarity, weak polarity, acidic, alcalic compounds).

1. Before elaborating the chromatographic separation methods, I wanted to investigate the chemical stability of charges. I considered it to be very important to use such stationary phase for HPLC separation that is able to provide a long-term reproducibility of analytical results.

2. I wanted to investigate the possible retention changes in the function of pressure, with high pressure drops characteristic of *non-porous* charges.

3. The HPLC separation of vitamins (both water- and liposoluble vitamins), analgesics and amino-acid derivatives are very important analytical tasks. However, characterization of such separation frequently requires long analysis time.
   The reason behind using *non-porous, micropellicular* reverse phases, was to achieve much shorter analysis time.

4. In terms of drug-analysis, the HPLC separation of optical isomers is very important as, normally, only one of the enantiomers have physiological effects. This is the reason why I wanted to elaborate a technique for the superfast separation of optical isomers by using *non-porous* charge.
   In terms of drug-analysis, the HPLC separation of optical isomers is very important. As it is well known only one of the enantiomers has physiological effects.

6. Due to the strong basic character of alkaloids, the separation of such compounds by a reverse phase chromatographic system is a critical analytical task.
   Nowadays the analysis of opium alkaloids from various matrices is an important HPLC separation task. Using this method the analysis of major opium alkaloids can be carried out from both the raw opium and the extract of the capsules of poppy.

My intention was to develop a kind of chromatographic separation which is suitable for the detection of both major and minor alkaloids.

This may bear importance for identifying the place of origin of a given sample.

RESULTS

In my work, I studied the applicability of *non-porous, micropellicular,* hydrophobic stationary phases with silica gel basis, for the high-performance liquid chromatographic separation of bioactive compounds. Although these stationary phases were developed for the separation of biopolimers, there are elaborated analytical methods to support the fact that they can be successfully used for the fast separation of compounds with small molecular weight (M<500) too. I selected such compound groups for the purpose of our analyses that have high significance in bio-sciences, in bio-medical researches, and in clinical tests.

1.) For liquid chromatography separations KOVASIL-C_{14} and KOVASIL-C_{18} columns were used. These columns were made by the Swiss based Chemie Uetikon AG.
The column charge was a high-purity (free from heavy metals), *non-porous, micropellicular,* silica gel with 1.5 μm particle-diameter.

To the surface of the particles of the charge an aliphatic hydrocarbon group of C_{14} and C_{18} chain length is connected by chemical binding.
A 33 x 4.6 mm column was filled with this hydrophobic, so-called reverse phase charge.

The chemical stability of this charge was studied. The results have shown an excellent chemical stability of the column. It also indicates that the column has a long life-cycle.

2.) The improvement of liquid chromatographic methods, as well as the increasing reproducibility of retention measurement, have driven my focus to the correlation between pressure and retention in liquid chromatography. When examining the relationship between pressure and retention I used measurements to verify the general validity of this correlation. I was the first to demonstrate that, among usually existing circumstances in chromatographic measurements, this significant retention-increasing effect is suppressed by the retention-reducing effect of the eluent getting warmer under the effect of the friction of the flowing eluent. I found a simple and clear correlation between the retention-changes and the changes in mole-volume of adsorbed/non-adsorbed molecules.

3.) The model mixtures for fast chromatographic separation, developed for bioactive molecules, were made of clean substances. The HPLC analysis of extracts made of various matrices (e.g. herbal, biological) may necessitate the modification of the circumstances used in the elaborated techniques (e.g. because of the disturbing effects of components retarding parallel with the chromatographic peaks of clean substances).

In the liquid chromatography separations performed by me, I developed a superfast separation process for the analysis of the following compound groups:

- water-soluble vitamins
- fat-soluble vitamins
- chiral compounds
- analgesics
- natural amino-acids
- alkaloids

The selected sample compounds are important not only from the aspect of practice. The other aspect that played a role in their selection was to verify the advantageous properties of *non-porous, micropellicular* charges through using compound groups that represent various different separation problems. Among the selected compounds there are compounds with strong polarity, weak polarity, acidic and basic chemical effects.

The time necessary for a separation is 1 to 3 minutes in general. The separation of amino-acids with 21 components can be carried out in 7 minutes. The fastness of separations, in itself, is advantageous for the chromatographic routine, but it has other beneficial impacts as well, for instance the low consumption of chemicals.

The shortness in analysis time means small-volume chromatographic peaks as well. The admixturing of samples is low compared to separations carried out in usual porous phases. Thus the traceability limit for the majority of compounds separated this way fell into the *femtomol* range.

The results I produced in the separation of opium alkaloids are significant, where both the separation speed and the sensitivity are considerably better than those that have been achieved so far.

DESCRIPTION OF A SELECTED RESEARCH RESULT

HPLC analysis of opium alkaloids

The separation and quantification of opium alkaloids is done in the most effective manner by using the reverse phase (RP) and high performance liquid chromatographic method (HPLC) with porous stationary phase. In the chromatographic analysis of raw opium and of „poppy straw", the collateral substances may interfere with the opium alkaloids to be identified, and therefore the determination of at least two major alkaloids are uncertain. The peak shape of late eluating components is wide and these problems, jointly, have impacts on the accurate determination of alkaloids. For these reasons those who perform liquid chromatographic assays for the alkaloids of poppy are forced to be satisfied with the quantitative determination of only four or, in certain cases, two major alkaloids.

From the results achieved through the quantitative and qualitative analysis of poppy-head extracts it is possible to conclude the place of origin and/or the place of production of the plant.

I have developed a new reverse-phase HPLC technique for the separation of the major opium alkaloids (morphine, codeine, thebaine, papaverine, and noscapine) by the application of a *non-porous, micropellicular* stationary phase. For the quantitative analysis I used brucine as our internal standard. Through the use of the gradient elution technique, I was able to perform the analysis of the herbal extract in 1.5 minutes.

Figure 2. shows the HPLC separation of an extract made of poppy-heads and containing the five major opium alkaloids (morphine, codeine, thebaine, papaverine, and narcotine) in a reverse-polarity (RP), *non-porous, micropellicular* stationary phase. Using this analysis method I could reduce the analysis time of opium alkaloids to 1.5 minutes so that I performed the separation at a temperature of 50 °C, and I used hepta-fluoro-butyric-acid (HFBA) for the generation of ion pairs. This way I achieved good separation for each and all opium alkaloids. The hepta-fluoro-butyric-acid has considerably changed the hydrophobic properties of the samples, the consequence of which was an increase in the components' retention. I achieved the optimum separation and the appropriate peak symmetry when I increased not only the strength of the solvent, but also the quantity of hepta-fluoro-butyric-acid, gradually, during the gradient process. I measured the traceability limits of the alkaloids. The results are shown in Table IV.

In the quantitative determination of alkaloids (Table II.) I calibrated the system for the five major alkaloids. In the measured concentration range the correlation coefficient between the concentration and the peak area was better than 0.9988 (Table I.).

As a comparison, Figure 1. shows the results of chromatographic assays performed on *non-porous* and *porous* stationary phases. As my results indicate, the separation is similar in both systems, but in the *non-porous* system the analyses can be be performed much faster and by orders of magnitude better sensitivity.

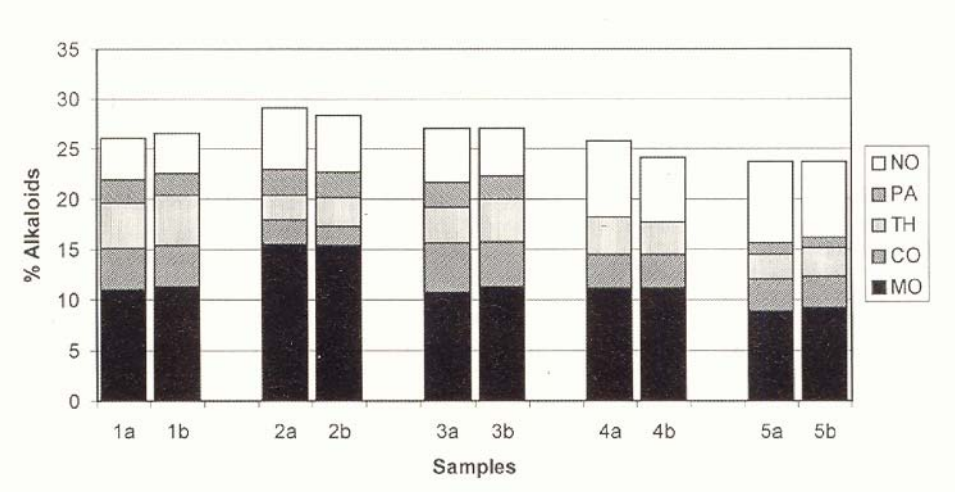

Figure 1 Comparison of the alkaloid content of five different opium samples as determined by HPLC on (a) *porous and* (b) *non-porous packing.* MO=morphine; CO=codeine; TH=thebaine; PA=papaverine; NO=noscapine

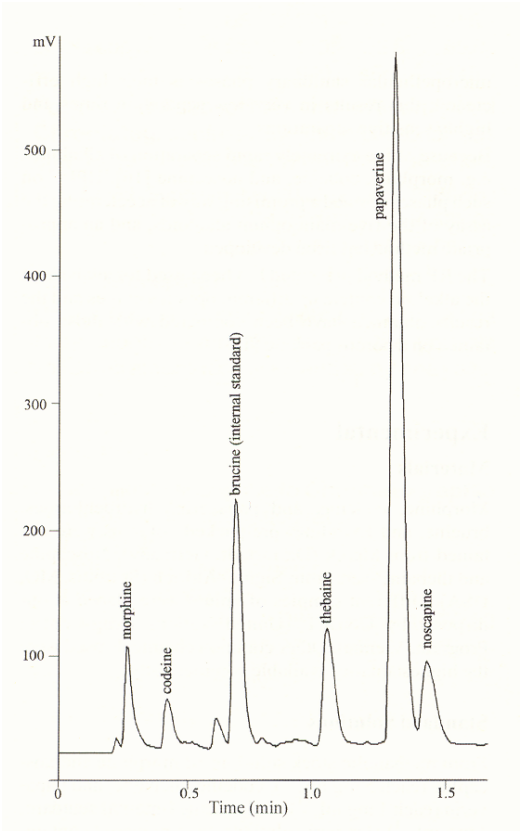

Figure 2. HPLC of gum opium; separation on Kovasil MS-C_{18} column

Table I. Calibration graph data.

Alkaloid	Linear range [mg ml^{-1}]	Correlation coefficient	Slope	Intercept
			(x, y, see below)	
Morphine	0.09-0.75	0.9988	0.132	0.0006
Codeine	0.03-0.28	0.9997	0.196	0.0000
Thebaine	0.02-0.21	0.9999	0.479	0.0032
Papaverine	0.01-0.20	0.9995	5.775	0.0055
Noscapine	0.11-0.34	0.9996	0.354	0.0094

Six calibraton points were plotted for each alkaloid.
x = amount of alkaloid (mg)
y = [amount of internalstandard (mg) × area of alkaloid peak] / area of internal standard peak

Table II. Reproducibility of the method for the major opium alkaloids

Alkaloid	Amount (%) in sample 1	Relative standard deviation (%)	Amount (%) in sample 2	Relative standard deviation (%)
Morphine	11.30±0.25	2.25	15.41±0.06	0.39
Codeine	4.07±0.10	2.56	1.89±0.05	2.44
Thebaine	5.02±0.06	1.16	2.87±0.03	0.97
Papaverine	2.17±0.04	1.86	2.52±0.05	2.00
Noscapine	3.98±0.04	0.95	5.61±0.09	1.56

Data are means from five determinations each (± standard deviation).

Table III. Reproducibility of the retention times of the major opium alkaloids

Alkaloid	Retention time, t_R (min)	Relative standard deviation (%)
Morphine	0.302±0.010	3.31
Codeine	0.429±0.015	3.50
Brucine	0.660±0.027	4.09
Thebaine	1.039±0.030	2.89
Papaverine	1.300±0.036	2.77
Noscapine	1.430±0.041	2.87

Data are means from 12 determinations (± standard deviation) performed during one week.

Table IV. Detection limits for the major opium alkaloids.

Alkaloid	Detection limit	Detection limit (ng)
Morphine	4.40 picomol	1.26
Codeine	5.70 picomol	1.71
Thebaine	1.05 picomol	0.33
Papaverine	35.9 femtomol	0.012
Noscapine	1.75 picomol	0.72

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Chromatographia (1999), 50, 75
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2. Ohmacht R., BOROS B., Kovács K.: High speed, high sensitivity separation of bioactive compounds by HPLC on non-porous phases
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3. Ohmacht R., BOROS B.: Effect of pressure on Solute Capacity Factor in HPLC Using a Non-Porous Stationary Phase
Chromatographia (2000), 51, S-205
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4. BOROS B., Kovács K., Ohmacht R.: Fast Separation of Amino Acid Phenylthiohydantoin Derivatives HPLC on Non-porous Stationary Phases
Chromatographia (2000), 51, S-202
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5. Krenn, L., BOROS, B., Ohmacht, R., Jelinek, L.: HPLC-Separation of Opium Alkaloids on Porous and Non-Porous Stationary Phases
Chromatographia (2000), 51, S-175
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Chromatographia (2002), 56, S-61
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1. Venue: XXth Days of Lectures on Chemistry, Szeged
   Date of presentation: 15th October 1997
   Title of presentation: *Extra rapid chromatographic separations*
   Authors: Borbála Boros, Róbert Ohmacht

2. Venue: MKE Analytic Department, Organic Analysis Specialised Group Meeting, Budapest
   Date of presentation: 11th November, 1997
   Title of presentation: *Rapid chromatographic separation of bioactive molecules (HPLC)*
   Authors: Borbála Boros, Róbert Ohmacht

3. Venue: Balaton Symposium ’97, Siófok
   Date of presentation: 4th September 1997
   Title of presentation: *Nonporous Silica Based Reversed Phase HPLC Packing. An Universal easy to use Alternative to Porous Adsorbents?*
   Authors: Róbert Ohmacht, Borbála Boros, Ibolya Kiss, László Jelinek
   Book of Abstracts: L-24 (page 48)

4. Venue: Southern Transdanubia Analytical Day, Kaposvár
   Date of presentation: 19th June, 1998
   Title of presentation: *Non-porous steady phases in liquid chromatography*
   Authors: Borbála Boros, Róbert Ohmacht

5. Venue: Separation Sciences Itinerary Congress ’98, Lillafüred
   Date of presentation: 1st October, 1998
   Title of presentation: *Retention changes occurring due to pressure alterations in liquid chromatography*
   Authors: Róbert Ohmacht, Borbála Boros

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   Authors: Ohmacht, R., Boros, B., Kiss, I., Jelinek, L
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   Date of presentation: 5th May, 1999
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   Authors: Borbála Boros, Liselotte Krenn, Róbert Ohmacht

8. Venue: HPLC´99 Granada (Spain)
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Authors: Krenn, L., Boros, B., Ohmacht, R.:
Book of Abstracts: L-28

11. Venue: Separation Sciences Itinerary Congress 2000
Date of presentation: 8-10th November, 2000
Title of presentation: *Recent results in the advancement of liquid chromatography packings*
Authors: Boros, B., Ohmacht, R.:
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Poster presentations in the topic

1. Venue of poster presentation: 4th Symposium on Instrumental Analysis, Graz
   Date of poster presentation: 20-23rd May, 1997
   Title of poster: *Separation of Drug Substances on Nonporous RP-HPLC columns*
   Authors: Borbála Boros, Róbert Ohmacht
   Symposium Abstracts: P36

2. Venue of poster presentation: Balaton Symposium '97, Siófok
   Date of poster presentation: 3-5th September, 1997
   Title of poster: *Fast Separation of Drug Substances on Nonporous RP-HPLC columns*
   Authors: Róbert Ohmacht, Borbála Boros, Krisztina Kovács
   Book of Abstracts: P-47 (page 125)

3. Venue of poster presentation: IV. Pharmanalysis Europe Conference, London
   Date of poster presentation: 27-28th October, 1997
   Title of poster: *Nonporous Silica Based Reversed Phase HPLC Packing. An Universal easy to use Alternative to Porous Adsorbents?*
   Authors: Róbert Ohmacht, Borbála Boros, Ibolya Kiss, László Jelinek
   Conference Proceedings: Part 15

4. Venue of poster presentation: Separation Sciences Itinerary Conference '98, Lillafüred
   Date of poster presentation: 30th September – 2nd October, 1998
   Title of poster: *Rapid chromatographic separation of essential amino acids*
   Authors: Borbála Boros, Róbert Ohmacht, Krisztina Kovács

5. Venue of poster presentation: Balaton Symposium '99, Siófok,
   Date of poster presentation: 1-3rd September, 1999
   Title of poster: *Effect of Pressure on Solute Retention*
   Authors: Boros, B., Ohmacht, R., Jelinek, L.
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6. Venue of poster presentation: Balaton Symposium '99, Siófok,
   Date of poster presentation: 1-3rd September, 1999
   Title of poster: *Quick Separation of PTH-Amino Acids*
   Authors: Boros, B., Kovács, K., Ohmacht, R.,
   Book of Abstracts: P-112

7. Venue of poster presentation: Balaton Symposium '01, Siófok,
   Date of poster presentation: 2-4th September, 2001
   Title of poster: *Simultaneous Determination of Oxidised and Reduced Glutathione*
   Authors: Boros, B., Ohmacht, R.,
   Book of Abstracts: P-92