# Fast liquid chromatography; potential of short, narrow bore reversed phase columns in pharmaceutical analysis

Ph.D. Thesis

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## 1, Introduction

Reducing analysis time and guarantying the quality of a separation in liquid chromatography (HPLC), requires high kinetic efficiency. The speed of analysis can be increased by different approaches. In liquid chromatography a new age has started with using sub-2 µm particles, monolith columns and shell particles.

On sub-2 µm particles, due to the narrow peaks, sensitivity and separation are improved at the cost of pressure. The analysis time could be reduced to a one or two minute interval without the loss of resolution and sensitivity [1-3]. Most commercial HPLC instruments have a maximum operating pressure limit of 400 bar, leading to the common practice of using short columns packed with small particles to speed up analysis [4,5]. A new nomenclature has come about with the term ultrahigh-pressure liquid chromatography (UHPLC). The first system for ultra-high pressure separation was released in the year of 2004. The new hardware was able to work up to 1000 bar and the system was called ultra performance liquid chromatography (UPLC$^{TM}$).

The concept of superficial or shell stationary phases, was introduced by Horváth and coworkers in the late 60s [6,7]. Horváth applied 50 µm glass bead particles covered with styrene-divinylbenzene based ion exchange resin and became known as pellicular packing material. Later on the core diameter was reduced and the thickness of active layer was cut to 0.5 µm and was used for fast separation of peptides and proteins [8]. Fused-core packing materials are commercially available in different diameters (5 µm, 2.7 µm, 2.6 µm and 1.7 µm). The 5 µm particles consist of a 4.5 µm nonporous core and a 0.25 µm porous silica layer, and the 2.7 µm particles consist of a 1.7 µm nonporous core and a 0.5 µm porous silica layer. The most recent shell stationary phase was released in the year of 2009 (2.6 µm and 1.7 µm particles). This Core-Shell$^{TM}$ technology produces particles, which consist of a 1.9 µm or 1.24 µm nonporous core and a 0.35 µm or 0.23 µm porous silica layer (respectively).

References

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## 2. Aim of the research

The development of a drug product is a very time consuming task, taking about 10–15 years from the synthesis of a lead compound to its commercialization. One of the main purposes of the pharmaceutical industry is to reduce this period of time by using high-throughput methods. Therefore, analytical laboratories of both development and routine analysis have to manage a large number of samples and must reduce the time response delivery during the drug development. One possible solution is to develop new quick and efficient separations for pharmaceutical analysis. With the aim of cutting analysis time and maintaining the separation efficiency, there has been important focus on high-speed chromatographic separations.

The aim of our research can be divided into three parts:

a) Critical evaluation of the capabilities and limitations of analytical columns, packed with sub-2 µm fully porous particles, compared to columns packed with 2-3 µm fully porous particles. Are the short narrow bore sub-2 µm columns really as efficient as it is cited so many times? Is the “small is beautiful” expectation valid?

b) Comparison of commercially available 5 cm long narrow bore columns packed with fully porous sub-2 µm particles and packed with sub-3 µm shell particles. Do the sub-3 µm shell particles really give the same kinetic efficiency as the fully porous sub-2

µm particles? If so, probably faster separations can be achieved with columns packed with core-shell particles due to their more advantageous permeability (larger particle diameter). Are there any significant parameters, which may affect the efficiency or only the core and shell ratio is the dominant?

c) Developing methods for pharmaceutical analysis (assay, impurity profiling, cleaning validation) within a day or even in a few hours? Is it possible to apply much faster systematic development by using short, narrow bore columns compared to conventional columns?

## 2. Equipment, software, columns

All measurements were performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler and a photo diode array detector (Waters Ltd. Budapest, Hungary). The UPLC system had a 5 µl injection loop and a 500 nl flow cell (path length = 10 mm). A polyether ether ketone (PEEK) tube (15 cm × 0.1 mm) is located between the column outlet and the detector. The overall extra-column volume ($V_{ext}$) is 12 µl as measured from the injection seat of the auto-sampler to the detector cell at 1 ml/min. The measured dwell volume is 130 µl. Data acquisition with a 80Hz data sampling rate and instrument control were performed by Empower 2 Software (Waters).

Calculation and data transferring to obtain the kinetic plots was achieved by using the Kinetic Method Plot Analyzer template (Gert Desmet, Vrije University Brussel, Belgium). The non-linear curve fitting to van Deemter and *h*-*v* plots was performed by using MS Excel (Solver). Method development was performed using DryLab 2010 chromatographic optimization software (Molnar-Institute, Berlin, Germany). The log*P* (octanol-water partition coefficients) values were predicted by ChemDesk (Medicinal Chemistry at your Desk), which is granted by Computer-Aided Design and Drafting (CADD) and was available at Gedeon Richter Plc.

Grace Vision HT C18 column with a particle size of 1.5 μm (50 mm x 2.0 mm) was purchased from Lab-Comp Ltd, Budapest. Column packed with 2.0 μm YMC UltraHT Pro C18 (50 mm x 2.0 mm) particles and Restek Pinnacle DB C18 1.9 μm (50 mm x 2.1 mm) columns were generous gift from Lab-Comp Ltd, Budapest. Shim-pack XR-ODS1 and Shim-pack XR-ODS2 columns with a particle size of 2.2 μm (50 mm x 2.0 mm) were purchased from Simkon Ltd, Budapest. Phenomenex Luna C18(2)-HST column packed with 2.5 μm particles (50 mm x 2.0 mm) and Gemini NX packed with 3.0 μm particles (50 mm x 2.0 mm) were purchased from GEN-Lab Ltd, Budapest. Thermo ODS Hypersil column packed with 3 μm particles (50 mm x 2.1 mm) and Hypersil Gold column packed with 1.9 μm particles (50 mm x 2.1 mm) were obtained from Bioszeparációs Technikai Ltd, Budapest. Waters UPLC$^{TM}$ BEH C18 column with a particle size of 1.7 μm (50 x 2.1 mm) was purchased from Waters Ltd, Budapest. Zorbax SB C18 column with a particle size of 1.8 μm (50 x 2.1 mm) was obtained from Kromat Ltd, Budapest. Fortis C18(2) 2.1 μm (50 mm x 2.1 mm) column was received from Lab-Comp Ltd for testing as a demo column. Ascentis Express C18 column (Supelco) with a particle size of 2.7 μm (50 mm × 2.1 mm) was purchased from Sigma–Aldrich Ltd. The Kinetex Core-Shell columns packed with 2.6 μm shell particles (50 mm × 2.1 mm, 100 mm x 2.1 mm, 100 mm x 3 mm, 100 mm x 4.6 mm and 150 mm x 3 mm) were obtained from GEN-Lab Ltd, Budapest. All of the columns were brand new (no other experiments were performed on them).

## 3. Results / Thesis

1) In our research data were collected (columns of eleven different vendors were investigated) proving that the efficiency of sub-2 μm particles is not as high as it can be expected. As the particle size is reduced, deviation of the real column efficiency from the expected efficiency is more significant.

2) Practically the same efficiency can be achieved with columns packed with any sub-2 μm porous particles independently from the particle diameter. The

efficiency loss may origin from the frictional heating and other adverse effects, which are serious at ultra-high pressure conditions.

3) The reduced plate height values show an increasing tendency as the particle diameter is decreased (Fig. 1.). The reason could be the difficulty in preparing a well-packed column bed of very fine particles. The obvious tendency in *A*-term of fitted *h*-*v* curves confirms this hypothesis.

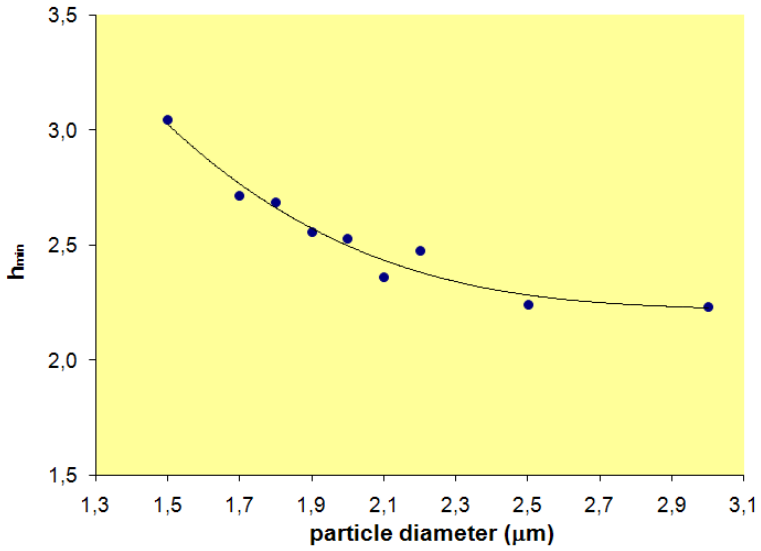


Fig. 1. reduced plate height minimum against particle diameter

4) 2.6 and 2.7 µm core-shell particles offer similar or better efficiency - and thus faster separation - at modest pressure than columns packed with fully porous sub-2 µm particles. The new Kinetex columns perform a very flat *C* term. Kinetex have a very narrow particle size distribution ($d_{90/10}$ = 1.15). Reduced plate height minimum values of $h_{min}$ = 1.9 (2.1 mm column), $h_{min}$ = 1.3 (3 mm column) and $h_{min}$ = 1.2 (4.6 mm column) were obtained. When 5 – 25 cm long columns are used, the Kinetex column provides the most favorable plate time values and offers the shortest analysis time ($N$ < 80000) of all commercially available columns (Fig.2.).

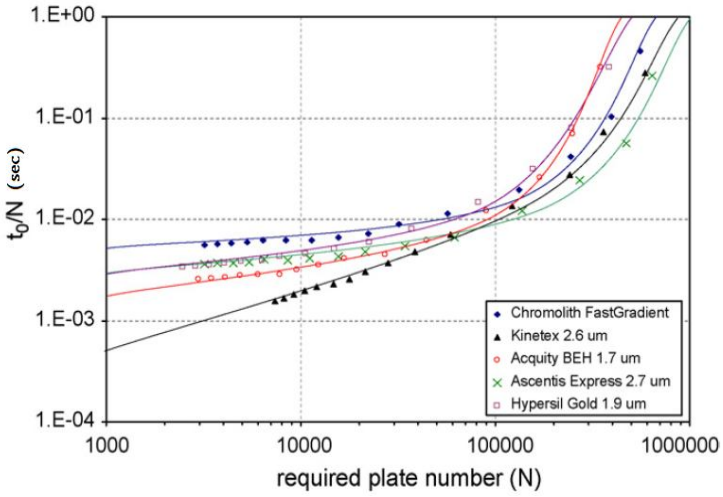


Fig. 2. Poppe plots of ivermectin. Experiments were conducted on 5cm long narrow bore columns in 95/5 ACN/$H_2O$, $\eta$ = 0.33 cPoise, at 35 $^{o}$C.

5) The narrow bore (2.1 mm) Kinetex column does not perform the expectation (Fig.3.). Complications associated with the packing of 2.1 mm Kinetex columns may be responsible for the increased minimum plate heights. The 3 mm and 4.6 mm diameter Kinetex columns resulted plate height minimum values of $h$ = 1.3 (3 mm column) and $h$ = 1.2 (4.6 mm column).

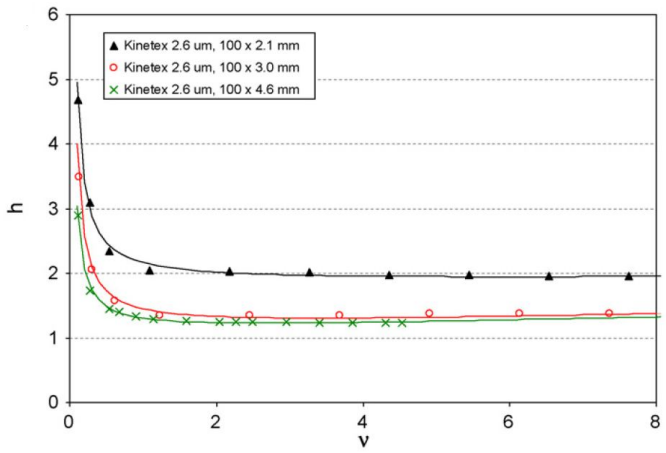


Fig.3. Experimental *h*-*v* plots of 2.6 µm shell-type (Kinetex, 100 mm x 2.1 mm, 100 mm x 3.0 mm and 100 mm x 4.6 mm), column (peak widths were corrected for the extra-column broadening). Mobile phase: 48 % acetonitrile – 52 % water, temperature: 35 $^{0}$C, injection: 0.5 µl, $D_M = 1.15 \times 10^{-5}$ cm$^2$/sec. Test analyte: estradiol.

6) The column packed with 2.7 µm fused-core particles gave unexpectedly high *C* terms. The surprising mass transfer characteristic of Ascentis Express particles can probably be explained with the differences of particle shape and external surface properties (Fig.4.). Another cause could also be that the mass transfer resistance is mainly controlled by the external film mass transfer resistance and not by the transparticle mass transfer.

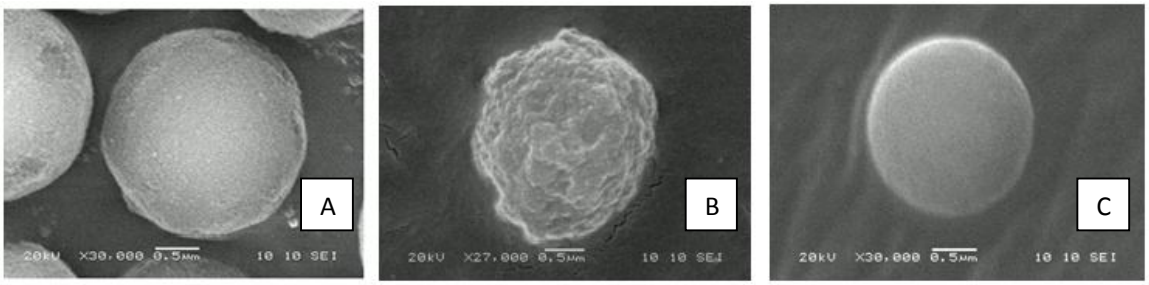


Fig. 4. SEM images of (A) Kinetex, (B) Ascentis Express and (C) Waters BEH particles

7) It is possible to develop methods for pharmaceutical analysis (assay, impurity profiling, cleaning validation) within a day or even in a few hours. If a 50 mm x 2.1 mm, sub-2 µm column is applied during the systematic method development, basic gradient runs with 7 and 21 minutes (at a flow rate of 0.4-0.5 ml/min) can provide reliable accuracy for the computer model simulation under ultra-high pressure conditions if gradient separation is necessary.

8) Based on our experiments we can state that DryLab separation modeling can be applied for elevated pressure (not only in HPLC practice) with high accuracy. The average of retention time errors did not exceed 5 % when the flow rate (pressure) was duplicated (p ~ 600 bar). When the flow rate was enhanced with a factor of 1.25 and 1.50 - compared to the flow applied for basic runs – the prediction error was approximately 3 % and 4 % (respectively). The total time saving of method development and utilizing the fast separation is approximately a factor of 3 - 5 compared to conventional method development and conventional LC analysis.

## 4. Papers, presentations

**Scientific papers (connected to the topic of the thesis)**

- Fekete, S., Fekete, J.

  Fast gradient screening of pharmaceuticals with 5 cm long, narrow bore reversed-phase columns packed with sub-3 μm core-shell and sub-2 μm totally porous particles,

  (2011) Talanta, Article in press, doi:10.1016/j.talanta.2011.01.053 (IF: 3.290)

- Fekete, S., Ganzler, K., Fekete, J.

  Simultaneous determination of polysorbate 20 and unbound polyethylene-glycol in protein solutions using new Core-Shell RP-HPLC column and condensation nucleation light scattering detection,

  (2010) Journal of Chromatography A, 1217, pp. 6258–6266 (IF: 4.101)

- Fekete, S., Ganzler, K., Fekete, J.

  Efficiency of the new sub-2 μm core-shell (Kinetex$^{TM}$) column in practice, applied for small and large molecule separation,

  (2011) Journal of Pharmaceutical and Biomedical Analysis, 54, pp. 482–490 (IF: 2.453)

- Oláh, E., Fekete, S., Fekete, J., Ganzler, K.

  Comparative study of new shell-type, sub-2 μm fully porous and monolith stationary phases, focusing on mass-transfer resistance

  (2010) Journal of Chromatography A, 1217, pp. 3642-3653. (IF: 3.756)

- Fekete, S., Ganzler, K., Fekete, J.

  Fast and sensitive determination of Polysorbate 80 in solutions containing proteins

  (2010) Journal of Pharmaceutical and Biomedical Analysis, 52 (5), pp. 672-679. (IF: 2.629)

- Fekete, S., Ganzler, K., Fekete, J.

  Facts and myths about columns packed with sub-3 μm and sub-2 μm particles

  (2010) Journal of Pharmaceutical and Biomedical Analysis, 51 (1), pp. 56-64. (IF: 2.629)

- Fekete, S., Fekete, J., Ganzler, K.
  Characterization of new types of stationary phases for fast liquid chromatographic applications
  (2009) Journal of Pharmaceutical and Biomedical Analysis, 50 (5), pp. 703-709. (IF: 2.629)

- Fekete, S., Fekete, J., Molnár, I., Ganzler, K.
  Rapid high performance liquid chromatography method development with high prediction accuracy, using 5 cm long narrow bore columns packed with sub-2 µm particles and Design Space computer modeling
  (2009) Journal of Chromatography A, 1216 (45), pp. 7816-7823. (IF: 3.756)

- Fekete, S., Fekete, J., Ganzler, K.
  Validated UPLC method for the fast and sensitive determination of steroid residues in support of cleaning validation in formulation area
  (2009) Journal of Pharmaceutical and Biomedical Analysis, 49 (3), pp. 833-838. (IF: 2.629)

- Fekete, S., Fekete, J., Ganzler, K.
  Shell and small particles; Evaluation of new column technology
  (2009) Journal of Pharmaceutical and Biomedical Analysis, 49 (1), pp. 64-71. (IF: 2.629)

- A summary of the paper entitled “Shell and small particles; Evaluation of new column technology” was published in Separation Science (2009), May, p. 12 (research roundup)

**Presentations (connected to the topic of the thesis)**

Oral presentations:

- Fekete, S., Fekete, J.: Recent possibilities of fast LC techniques (National Separation Science Meeting, 2010, Tapolca, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: Fast RP-HPLC techniques in the field of bio-similar formulation development (Pharmaceutical Analytical Symposium, 2010, Siófok, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: Efficiency of the new Core-Shell columns (Chromatographic Seminar, 2010, Szeged, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: UHPLC; Does the practice always meet the theory (Balaton Symposium, 2009, Siófok, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: Efficiency and applications of Fused-Core columns; Fast separations (Chromatographic Seminar, 2009, Szeged, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: Efficiency of shell and totally porous particles (National Separation Science Meeting, 2008, Sárvár, Hungary)

- Fekete, S., Ganzler, K.: Fast LC for pharmaceutical cleaning control analysis (Hungarian Chemists' Society Meeting, 2007, Budapest, Hungary)

Poster presentations:

- Oláh, E., Kiss K., Fekete, S., Fekete, J., Ganzler, K.: Application possibilities for fast chromatographic methods in UHPLC and HPLC systems (Balaton Symposium, 2009, Siófok, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: Efficiency Characterization of new types of stationary phases for fast liquid chromatographic applications (Balaton Symposium, 2009, Siófok, Hungary)

- Fekete, S., Ganzler, K., Fekete, J.: General UHPLC method for pharmaceutical cleaning control analysis (National Separation Science Meeting, 2008, Sárvár, Hungary)

- Fekete, S., Ganzler, K.: Small particles, or „Shell" particles? Comparison of rapid techniques in application of steroid separation (Csaba Horváth Medal Award Symposium, 2008, Insbruck, Austria)

- Fekete, S., Ganzler, K.: Introducing and comparing rapid HPLC techniques in pharmaceutical cleaning control and validation analysis (Csaba Horváth Medal Award Symposium, 2008, Insbruck, Austria)

- Fekete, S., Ganzler, K.: Fast analysis of steroids on zirconium-dioxide based stationary phases at elevated temperature (Balaton Symposium, 2007, Siófok, Hungary)

- Fekete, S., Ganzler, K.: Comparison of rapid HPLC techniques applied in pharmaceutical analysis (HPLC 2007, 2007, Ghent, Belgium)