University of Szeged
Doctoral School of Pharmaceutical Sciences

Programme of Pharmaceutical Analysis

Programme director: Prof. György Dombi Ph.D., C.Sc.

Institute of Pharmaceutical Analysis

Supervisors:

Prof. György Dombi, Ph.D., C.Sc.

Gerda Szakonyi, Ph.D., Pharm. D.

Éva Kalmár, Pharm.D.

CHALLENGES OF HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE ASSAY OF COMBINED DRUG PRODUCTS

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1. Introduction and aims

Pharmaceutical analysis is one of the most challenging fields of analytical chemistry. Pharmaceutical analysts carry out the qualitative and quantitative control of APIs and drug products and also develop and validate appropriate methods. These methods are routinely used by manufacturing companies in process testing and by authorities for the quality control of drug products. In the vast majority of pharmaceutical analyses, instrumental analytical methods are applied. The most widespread of all techniques is HPLC, which is complemented or hyphenated with mass spectrometry, spectrophotometry, NMR or others. In consequence of its dominant role in the pharmaceutical industry, HPLC is developing with huge leaps nowadays. UHPLC is increasingly making conventional HPLC obsolete. The field of core-shell particles, the application of new detection techniques or 2D chromatography and the very popular hyphenated systems provide many interesting problems or challenges. Nevertheless, it should not be forgotten that these development directions are very cost-intensive, as up-to-date instruments and even columns are very expensive. Smaller national pharmaceutical companies and state-financed control laboratories of national authorities therefore cannot always follow the development of instrumental analysis in this direction. One of my main goals was to develop modern,

rapid, precise and reproducible, but also cost-effective HPLC assay methods which are generally available and applicable for most users.

The development of sample preparation from complex drug products is the most challenging area of assay method development for HPLC. To demonstrate this, I have chosen to show two examples in my thesis. In the first example, the development problem relates to the separation of three physico-chemically different APIs of a multicomponent drug product. In the second example, the challenge is the complete recovery of the API from various complex suppository dosage forms manufactured with different bases.

Even today a significant number of suppositories are prepared extemporaneously in Hungary. Most are prepared by clinical pharmacies for paediatric use. The magistral preparation of suppositories is cheap; moreover, customized personal therapy can be achieved much better through their use. On the other hand, the independent quality control of such products by authorities is not carried out at present. Accordingly, I would like to stress here how important this topic is and, by demonstrating the consequences of technological errors that may be committed during preparation, I would like to contribute to improving the

quality of extemporaneous pharmaceutical manufacturing in pharmacies.

2. Methods

Instruments and other equipment

HPLC measurements were carried out on a Shimadzu Prominence UHPLC system (Shimadzu Corp., Kyoto, Japan) equipped with an LC-20AD pump, a 4-port solenoid mixing valve, a CTO-20A column oven, a DGU-20ASR degasser, and an SPD-M20A UV/VIS PDA detector with a 10 mm optical path length flow cell. Samples were injected via a Rheodyne 6-port manual injector valve fitted with a 20 µl sample loop. Separation was studied on a Hypersil ODS (C18) 150x4.6 mm, 5 µm column (Thermo Scientific, Keystone, UK), a Luna C18(2), 150x4.6 mm, 3 µm column (Phenomenex, Torrance, CA, USA) and a Zorbax SB-C18 150x4.6 mm, 3.5 µm column (Agilent, Santa Clara, CA, USA) during the method development procedure. Data acquisition and peak integration were carried out with LCSolution (Shimadzu Corp., Kyoto, Japan) chromatographic data acquisition and processing software. The results were evaluated with LC Solution and Microsoft Office Excel 2007 software. The log D vs. pH functions for the tested compounds were predicted with Pallas intelligent chromatographic software.

Spectrophotometric measurements were carried out on a Shimadzu UV-1601 UV/VIS double-beam spectrophotometer. Throughout the measurements, quartz cells with 10 mm optical path length were used. The spectrophotometric data were evaluated with Microsoft Excel.

^{1}H NMR spectra were recorded on a BRUKER Avance DRX 500 spectrometer at room temperature, with a deuterium lock. There was no water suppression during the experiment. The carrier frequency (O1) was placed at 7.01 ppm and a 16.00 ppm wide region was detected, the excitation was carried out with a 30° pulse (PW_{90}=12.5 μs), the interpulse delay was set to 3 seconds, the acquisition time was 2.05 s and 8 transients were collected into 32K data points. The spectral processing included an exponential filtering with 0.3 Hz, zero-filling to 64K data points and a complex Fourier transformation. The data collection and data processing were carried out with Bruker XWIN-NMR 3.1 software.

During the sample preparation for cerimetric titration, one suppository was melted over a 40 °C water bath and 3 replicate samples of 0.20-0.30 g were weighed from the molten mass into titration flasks. 10.0 ml of 15% sulfuric acid was added to each sample and the mixture was heated to 40 °C to extract the API from the suppository base. The mixture was then cooled to room

temperature, 15 ml of distilled water was added, and after mixing and the addition of 1 drop of ferroin indicator, titration with 0.05 M cerium(IV) sulfate volumetric solution was performed until the colour of the solution changed from orange to green and remained green for at least 1 min.

3. Results

Part I. Development and validation of HPLC assays

1. A HPLC method was developed and validated for the simultaneous assay of an oral powder dosage form containing 3 APIs.
   1.1. The pH of the mobile phase was determined with chromatographic prediction software on the basis of paracetamol, acetylsalicylic acid (ASA) and papaverine log D vs. pH functions. pH 3.4 ±0.05 was found to be a reasonable compromise for the pH of the aqueous phase.
   1.2. Appropriate organic-aqueous ratio and gradient profile were determined in order to achieve satisfactory retention and peak shape.
   1.3. Different stationary phases were compared for the separation of compounds with different polarities. Of the 3 stationary phases tested (ODS Hypersil, Luna C18, Zorbax SB-C18), Zorbax SB-C18 proved to be the most suitable

on the basis of the separation parameters calculated from the chromatograms.

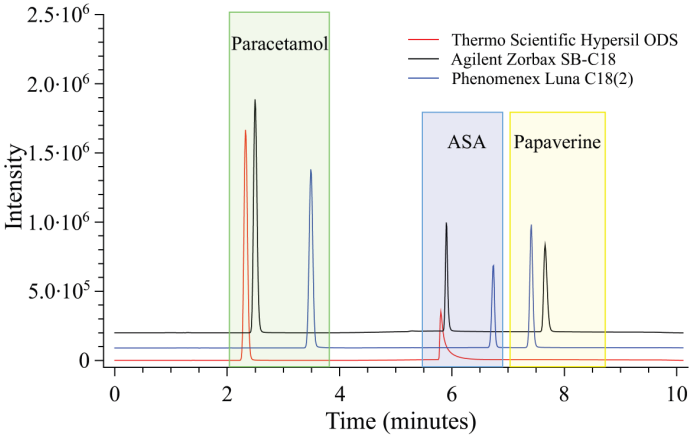

Figure 1 Comparison of the three stationary phases. It can be observed that papaverine was completely retained on Hypersil ODS

1.4. The developed method was validated by testing linearity, precision (repeatability and intermediate precision), accuracy, specificity and robustness. The method met all the acceptance criteria set up before validation.

2. A HPLC method was developed and validated for the assay of aminphenazone (AMFZ) and paracetamol.

2.1. A RP-HPLC assay method was developed for AMFZ.

2.2. A RP-HPLC assay method was developed for paracetamol.

2.3. Both methods were validated by testing linearity, precision (repeatability and intermediate precision), accuracy, specificity and robustness. Accuracy was tested in an extended range (up to 450% of the labelled claim) in the case of AMFZ, and specificity was also tested with respect to the solvent and matrix components. Both methods met all the acceptance criteria set up before validation.

Part II. Challenges in the development of sample preparation for suppositories

3. A sample preparation procedure was elaborated for the analysis of suppositories prepared from any of the possible 3 different suppository bases.

3.1. API recovery from lipophilic hard fat suppository base with a freezing technique.

3.2. API recovery from hydrophilic massa macrogoli suppository with dissolution.

3.3. API recovery from W35TT suppository base with micelle breaking. The effects of solvent pH, salt concentration and length of US treatment on the recovery efficiency were studied independently and simultaneously. The most effective conditions were found to be the combination of 100 mM NaCl, 40 mM NaOH and 30 min US treatment.

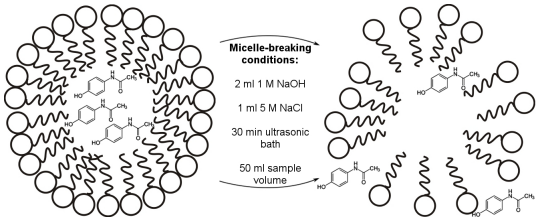

Figure 2 Theoretical figure of micelle-breaking mechanism

3.4. Turbidimetric CMC determination of the surfactant components of W35TT suppository base. Tween 20, Tween 60 and Tween 20+60 CMC were measured in MeOH-water as solvent. Tween 20+60 CMC was measured in MeOH-water and under the most effective sample preparation conditions.

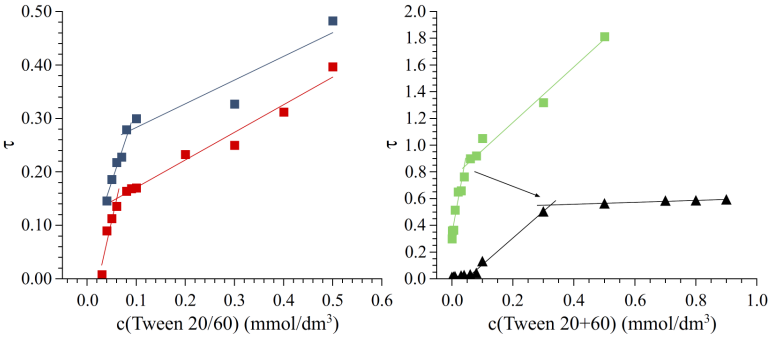

Figure 3 Turbidimetric plots for determination of CMCs of Tween 20 (▪), Tween 60 (▪), Tween 20 & 60 (▪) and Tweens 20 & 60 with salt and base (▪)

3.5. In a comparative dissolution study of adeps solidus and W35TT-based suppositories, dissolution samples were

analysed with the developed HPLC method. Dissolution was found to be faster from the W35TT matrix in 900 ml of dissolution medium (pH=7.5). The beneficial effect of surfactants observed under *in vitro* conditions may decrease when an *in vivo* available volume of rectal fluid of about 2 ml is considered. In this case, the concentration of applied surfactants will be higher than the CMC. Thus, formation of micelles cannot be avoided, which may decrease the bioavailability of the administered drug. The application of less surfactant is therefore suggested.

3.6. The structural stability of AMFZ and paracetamol was studied with NMR spectroscopy under the conditions applied for micelle breaking. (100 mM NaCl, 40 mM NaOH and 30 min US). No change was observed between the NMR spectra of the reference and test solutions, which proved that the 2 APIs did not suffer degradation during US and basic treatment.

Part III. Quantitative analysis of magistrally produced suppositories

4. Comparative analysis was performed of magistrally produced suppositories (HPLC assay) with identification of manufacturing errors.

4.1 The assay results obtained with cerimetric titration and RP-HPLC were compared. Classical titration and HPLC assay can be applied equivalently for the analysis of AMFZ.

4.2. In a dosage uniformity study of magistrally produced suppositories, sample batches of 10 AMFZ-containing suppositories prepared by and purchased from 15 Hungarian pharmacies were tested.

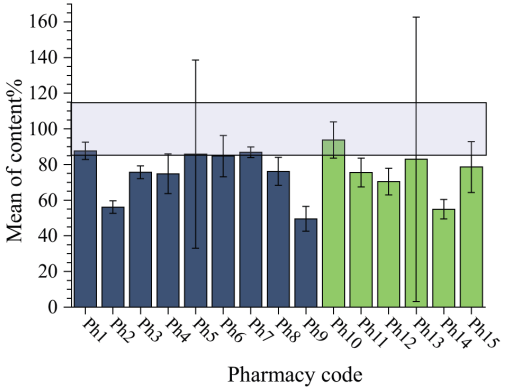

Figure 4 Mean API contents for the samples, with the standard deviations. Samples Ph1-Ph9: measured by HPLC; samples Ph10-Ph15 measured by cerimetric titration

4.3. The effects of *f* on the assay results were identified. It is strongly suggested that the *f* values for the most common APIs should be determined, and applied in everyday pharmaceutical practice.

4.4. The effects of stirring on the homogeneity and average assay of the suppository samples indicated that the lack of stirring may lead to decreased homogeneity and API content, depending on the phase in which stirring was omitted.

4. Conclusion

Conclusions of Part I

The presented results clearly demonstrate that the most challenging part of the development was to find an appropriate stationary phase on which all 3 compounds can be separated with good peak symmetry and resolution. The Hypersil ODS stationary phase proved to be too retentive for papaverine and it was obvious during the development that good peak shape cannot be achieved. The application of a stationary phase equivalence chart led us to Zorbax SB-C18 and Luna C18 stationary phases, which were more hydrophobic and more selective according to the chart data. The increased hydrophobicity of the stationary phase made it necessary to reduce the final organic modifier content of the gradient. In this way, all three compounds eluted within 10 min and were separated well on both stationary phases. Another problem was the low solubility of ASA in water. In organic solvents such as MeOH or ACN it is freely soluble, but a higher

organic content of the mobile phase would have caused the too early elution of paracetamol (within the void peak) which is unacceptable. A too low organic content, on the other hand, led to the ASA precipitating and clogging the tubing and the column. In the final method, a balance was successfully found between retention and solubility by applying 7% ACN content in the initial phase of the gradient. The peak symmetry and selectivity were found to be better on the Zorbax SB-C18 phase. This column was therefore chosen for the final method and the validation steps were carried out with this phase. An elevated column temperature made it possible to develop a rapid and efficient method with rather low back-pressure (a maximum of about 100 bar during the runs), which ensures a longer column lifetime. The method validation was carried out according to current ICH guidelines. All the results satisfied the guideline requirements.

Conclusions of Part II

The data presented in this section revealed that a rapid, efficient and robust sample preparation procedure and HPLC method were successfully developed and fully validated for the routine quality control of the dosage units of suppositories containing AMFZ as active substance in various vehicles as supporting materials. The method is simple and sufficiently

general to be conveniently used for the regular quality control of AMFZ suppositories formulated through the use of different suppository bases.

An adequate sample preparation method was developed for Tween 20 and Tween 60-containing hard fat-based suppositories. We proved that micelles are formed in the sample solution and successfully destabilized them by applying 100 mM sodium chloride and 40 mM sodium hydroxide and 30 min US treatment, which made the complete release of two physico-chemically different APIs possible. Provided that there are appropriate chromatographic methods at hand, the developed sample preparation method could be utilized for the determination of other drug molecules from surfactant-containing suppositories too. This problem raises the question of how micelle formation influences drug release during therapeutic application. As the problem appeared during the melting of the suppository for analytical sample preparation, the question arises of whether this phenomenon would cause homogeneity problems at surfactant concentrations higher than the CMC in suppositories produced by industrial technology, which is based mainly on moulding techniques for large quantities.

Conclusions of Part III

The results for paediatric suppositories produced extemporaneously under predefined conditions in Hungarian independent pharmacies revealed that serious errors may arise if the rules used in the pharmaceutical technology for preparing such suppositories are not strictly adhered to, and the assay results on the individual dosage units may be affected. On the other hand, suppositories prepared with strict adherence to the correct manufacturing practices conform to the specifications described in EP 7.8. Since extemporaneously prepared suppository preparations are frequently compounded and supplied in central European clinical pharmaceutical practice because of the low costs involved, I would encourage the use of and the inclusion of the *f* values for the most common APIs and for the most common suppository bases into the European or national pharmacopoeias. To my knowledge, the paucity of this information prohibits the preparation of the “right” dose for the “right” patient and may even cause harm. Calibration of the mould and the determination of the *f* value for these basic common suppository bases can be accomplished very simply.

5. Publications

Full papers related to the thesis

É. Kalmár, K. Ueno, P. Forgó, G. Szakonyi, G. Dombi
Novel sample preparation method for surfactant containing suppositories; effect of micelle formation on drug recovery
Journal of Pharmaceutical and Biomedical Analysis 2013 (83) 149-156

IF: 2.947*

É. Kalmár, J. Lasher, T. Tarry, A. Myers, G. Szakonyi, G. Dombi, G. Baki and K. Alexander
Dosage uniformity problems which occur due to technological errors in extemporaneously prepared suppositories in hospitals and pharmacies
Saudi Pharmaceutical Journal, accepted for publication

IF: 0.954*

É. Kalmár, A. Gyuricza, E. Kunos-Tóth, G. Szakonyi, G. Dombi
Simultaneous quantification of paracetamol, acetylsalicylic acid and papaverine with validated HPLC method
Journal of Chromatographic Sciences, accepted for publication

IF: 0.749*

É. Kalmár, B. Kormányos, G. Szakonyi, G. Dombi
Validated HPLC determination of 4-dimethylaminoantipyrine in fundamentally different suppository bases
Indian Journal of Pharmaceutical Sciences, accepted for publication

IF: 0.338*

* 2012 data

Scientific lectures related to the thesis

É. Kalmár:
Kromatográfiai technikák - Gyógyszerfejlesztés analitikai problémái
QP3 Továbbképzés
16. April 2013, Szeged, HU (lecture)

É. Kalmár:
Tenzid tartalmú kúpok analitikai problémái és megoldásai
KEN XXXV. Kémiai Előadói Napok
29-31. October 2012, Szeged, HU (lecture)

É. Kalmár, B. Kormányos, G. Szakonyi, G. Dombi
Fast efficient and robust UHPLC determination of 4-dimethylaminoantipyrine from different types of suppository vehicles
4th ISMCK International Student Medical Congress
21-24. June 2012, Košice, SK (lecture)

É. Kalmár, B. Kormányos, G. Szakonyi, G. Dombi
Fast and robust HPLC method for aminophenazone assay from distinct suppository bases
TÁMOP- From molecule to drug
24-25. May 2012, Szeged, HU (poster)

Kalmár É.:
Aminofenazon tartalmú magisztrális gyermekkúpok hatóanyagtartalmának ellenőrzése
X. Clauder Ottó Emlékverseny
13-14. October 2011, Budapest, HU (lecture)