# Application of modern separation techniques for solving practical problems encountered by an official medicines control laboratory

Abstract of Ph.D. Thesis

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## Introduction

The primary goal of drug discovery and production is the promotion of human welfare by eliminating the causes of diseases, reducing symptoms or improving the resistance of the body. The three major pillars of drug development are efficacy, safety and quality. The latter one is particularly important since it is a prerequisite of the two other characteristics. Constant assurance of an appropriate quality of medicinal products is a major issue in healthcare and the responsibility of the competent authorities is essential in this field.

The National Institute of Pharmacy (NIP)[*] as the official drug control agency for human medicinal products in Hungary has a wide range of duties. The official medicines control laboratory (OMCL) working as part of NIP's Quality Evaluation and Control Unit carries out various tests ranging from proficiency studies launched in order to control the laboratory's quality standards through market surveillance tests up to investigation of reports of adverse reactions and of suspected quality defects. Furthermore, besides approved medicinal products and individual extemporaneous preparations produced by pharmacies falsified and/or illegal medicinal products have emerged as an undesired category of growing importance.

As a result of these diversified activities elaboration of new analytical methods is often unavoidable. Due to their selectivity, readiness for automatisation, versatility and the permanent dynamic innovation in this area separation techniques play a fundamental role in pharmaceutical analysis. In addition, hyphenated techniques (such as high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) and/or nuclear magnetic resonance (NMR) spectroscopy) which have become a part of routine work recently have opened new horizons for structure elucidation and quantification studies.

## Aims

The major objective of the thesis is to give examples of possible application of separation techniques in drug control testing by presenting three novel analytical methods developed using different techniques (i.e. capillary electrophoresis (CE), HPLC and HPLC-MS). Each analytical method was elaborated and validated in order to solve practical problems encountered by our laboratory.

1. The first aim of our work was to develop a fast and simple capillary zone electrophoretic method for determination of the active pharmaceutical ingredient (API) content and the level of its major degradation product (pyridine analogue) in tablets containing amlodipine besilate. The method was intended to be used in market surveillance studies of approved tablets produced by different manufacturers. The preliminary expectations of the method were to be fast, simple, cost-effective and to have a limit of quantification (LOQ) not greater than half of the specification prescribed in the European Pharmacopoiea (0.3 per cent). Furthermore, comparable performance parameters to those of the corresponding HPLC methods described in the Ph. Eur. monograph were set as a minimum requirement. To evaluate this, we also carried out a comparison of the optimized CE method and the Ph. Eur. HPLC tests.
2. In association with an adverse reaction (suspected intoxication) reported to the NIP following administration of an individual extemporaneous preparation to a 13-month-old infant we aimed to develop a method for simultaneous assay of theophylline,

[*] Since 1 May 2011 the NIP continues to function as part of the National Institute for Quality- and Organizational Development in Healthcare and Medicines which, however, does not affect NIP's current activities.

phenobarbital, codeine and ephedrine by HPLC in a suppository. Because the expert opinion issued by the NIP strongly influenced the judgement about possible responsibility of the pharmacist, particular emphasis was put on the validation of results. The minimum requirement set for the method was to produce results enabling unambiguous establishment of compliance/non-compliance of the preparation with respect to content specifications so that a possible quality defect as a cause of intoxication could be confirmed/ruled out.

3. The final part of our work was related to powder samples identified as psychotropic cathinone derivatives which were submitted for expert opinion by the Hungarian Customs and Finance Guard with the suspect of illegal distribution of medicinal products. Our goal was to elaborate an HPLC-MS/MS target screening procedure which would allow rapid and simple detection and identification of the cathinone derivatives mephedrone, flephedrone, butylone, MDPV, methylone and methedrone. A method like this may ease and speed up the work of the laboratory which is especially important considering the usually rather strict deadlines required in case of expert opinions. Our intention was to design the method such that it could be used as a basis for future quantification tests. For information, limit of detection (LOD) was also determined.
   During elaboration of the target screening method another sample presumed to be a new cathinone derivative according to preliminary tests was submitted to the laboratory for analysis. Therefore, identification and a detailed structure elucidation of the molecule by HPLC-MS/MS, high resolution time-of-flight (TOF-)MS as well as NMR and infrared (IR) spectroscopy was set as an objective. In addition, purity tests and identification of the salt form were carried out and applicability of the HPLC-MS/MS screening method to the newly identified substance was examined.

Besides presenting new scientific results we also intended to demonstrate by this compilation of examples the differences of the approaches used in the management of these essentially dissimilar tasks.

## Methods

*Market surveillance testing of tablets containing amlodipine besilate by CE*[1]

CE analysis was performed on a HP G1600AX 3DCE instrument equipped with a diode array detector (DAD). Separation was carried out in an extended light path uncoated fused silica capillary with a full length of 64.5 cm, an effective length of 56 cm, an internal diameter of 50 μm and a bubble cell diameter of 150 μm. Temperature was maintained at 25.0 °C. Detection wavelength was 223 nm and the applied voltage was 30 kV. Samples were introduced hydrodynamically applying 300 mbar·s. A 8:2 *V/V* mixture of 25 mM citrate buffer adjusted to pH 7.0 and methanol was used as background electrolyte.

Sample preparation consisted of mechanical shaking of the appropriate number – depending on whether assay or monitoring of the degradation product was carried out – of powdered tablets in a 1:1 *V/V* mixture of 40 mM citrate buffer (pH 7.0) and methanol for 15 minutes, then in an ultrasonic bath for 5 minutes and subsequent centrifugation. The clear supernatants were used for analysis.

For HPLC tests an Agilent 1200 Series RRLC instrument was applied. Tests were performed using a Zorbax XDB C18 column as prescribed in the 6$^{th}$ Edition of Ph. Eur. Sample preparation was carried out as described in the corresponding marketing authorisation documents.

*Simultaneous assay by reversed phase ion pairing (RP-IP)-HPLC of theophylline, phenobarbital, codeine and ephedrine in an extemporaneous suppository*[2]

A HP 1050 LC instrument was used for analysis. Compounds were separated on a Purospher Star RP-18e 250×4.6 mm column at 28 °C using water-acetonitrile gradient elution at a flow rate of 1 $mL \cdot min^{-1}$ and with an injection volume of 50 µL. 5 mM sodium octane sulfonate was applied as ion-pairing agent and acetic acid acetic acid to maintain an acidic pH. Detection wavelengths were set as follows: 272 nm for theophylline, 250 nm for phenobarbital, 285 nm for codeine and 256 nm for ephedrine.

Samples were subjected to a multistep sample preparation procedure before chromatographic separation. Three parallel samples of the homogenised suppositories were accurately weighed and dissolved in approximately 3 ml dichloromethane. The solutions were rinsed into an extraction funnel with further 2 ml dichloromethane. After mechanical shaking of each sample with 3×5 ml acetate buffer adjusted to pH 5.0 for 3×5 minutes at 150 rpm aqueous layers were collected in 50 mL volumetric flasks. In the next step the remaining organic layers were shaken with 3×5 mL 0.1 M NaOH solution for 3×10 minutes at 150 rpm. The aqueous layers were then added to the collected acetate buffer layers and the solutions were diluted to 50.0 mL with the acetate buffer. Finally, 10.0 mL of each of the extracts were diluted to 25.0 mL with mobile phase A (water + acetonitrile + acetic acid 90:10:2 *V/V* containing 5 mM sodium octane sulfonate) and the diluted solutions were filtered through a 0.45 µm membrane filter.

*Target screening of cathinone derivatives in unknown powder samples by HPLC-MS/MS; detection and identification of the new cathinone derivative 4'-methylethcathinone (4-MEC)*[3]

The following equipment was used. HPLC-MS/MS: Agilent 1200 series HPLC equipped with a UV-VIS DAD + Agilent 6410A Triple Quad MS (ion source: MMI). FT-IR: Perkin Elmer Spectrum 400 FT-IR/NIR spectrophotometer. NMR: Varian VNMRS spectrometer 600 MHz; internal standard: DSS. TOF-MS: Agilent Technologies 1260 Infinity HPLC + Agilent 6230 Time of Flight MS equipped with a Jet Stream™ ion source; reference masses: *m/z* 121.050873 and 922.009798; software: Agilent MassHunter Workstation Software B.01.03.

Methedrone and methylone hydrochloride reference standards were purchased from LGC Standards. Identification and purity testing of mephedrone, flephedrone, butylone and MDPV hydrochloride salt samples used as reference standards were carried out by IR, high resolution MS, NMR and HPLC-UV-MS/MS techniques. For the optimised HPLC-UV-MS/MS method substances were separated on a Zorbax Eclipse XDB $C_{18}$ (3.5 µm) 75×4.6 mm column. Column temperature was maintained at 25 °C. A water-acetonitrile gradient was applied at a flow rate of 0.6 $mL \cdot min^{-1}$. 0.1 per cent formic acid was added to the mobile phase to enhance ionisation and to control pH. UV chromatograms were recorded at 210 nm while UV spectra were stored in the 210–400 nm range. MS ion source was operated in ESI(+) (electrospray ionisation with positive polarity) mode and quadrupoles in multiple reaction monitoring (MRM) mode. Compound dependent MS parameters for each molecule were optimised with respect to maximum efficiency of ionisation and fragmentation by adjusting fragmentor voltage and collision energy. For this purpose flow injection analysis was (FIA) used. Parameter settings and transitions resulting in the most abundant responses were chosen for analysis.

## Results

*Market surveillance testing of tablets containing amlodipine besilate by CE*

The following parameters were optimised during method development: pH and composition of the background electrolyte, temperature, internal diameter of capillary, detection wavelength, injection. Procaine hydrochloride was used as internal standard because its chemical structure shares several common features with those of amlodipine and the pyridine analogue, its p$K_a$ value is practically the same as those of the two analytes, the UV absorption maximum at 223 nm is ideal for detection, it is cheap, readily available and non-toxic. To minimise electrodispersion composition of solvent mixture applied for extraction was chosen such that ionic strength and pH be possibly similar to the background electrolyte.

The method was validated in accordance with the corresponding quality assurance document of the OMCL network based on ICH Guideline Q2(R1). Selectivity assessment consisted of excluding possible interference due to excipients and residual process-related impurities or intermediates described by the actual Ph. Eur. monograph. LOD and LOQ of the impurity assay were 0.01 and 0.04 per cent, respectively, expressed as percentage of amlodipine besilate content. Thus, the minimal requirement of an LOQ greater than 0.15 per cent was met. Accuracy and precision was determined by spiking the product of one of the manufacturers with known amounts of API and impurity. Results complied with the usual requirements established for assays and purity tests. Robustness of the method was demonstrated by carrying out suitable tests.

Tablets produced by three different manufacturers were tested using both the newly developed and validated CE method and the corresponding HPLC tests prescribed by Ph. Eur. All products complied with the specifications. After comparison of the results obtained with the two methods, CE analysis appeared to be equivalently suitable for market surveillance of amlodipine tablets as the official HPLC methods. However, measurement uncertainty expressed as standard deviation appeared to be significantly greater in case of CE (*F*-test; $P = 0.95$).

*Simultaneous assay by RP-IP-HPLC of theophylline, phenobarbital, codeine and ephedrine in an extemporaneous suppository*

"Related substances" test included in the monograph "Codeine hydrochloride monohydrate" of Ph. Eur. 6$^{th}$ Edition was used as a basis for development of the chromatographic method. Separation of the 4 compounds in a single system required the use of gradient elution where retention times were as follows: 5.3 min for theophylline, 22.2 min for phenobarbital, 25.1 min for codeine, 31.4 min for ephedrine. A stepwise gradient was used instead of a linear profile since this practically enabled separation of the three later eluting compounds under isocratic conditions. This is generally considered to be beneficial to reproducibility of the chromatograms and precision of the assay.

Extraction process of the active substances was optimised taking into account the p$K_a$ values. Dichloromethane proved to be suitable for dissolving the suppository basis. Extraction with an acetate buffer adjusted to pH 5.0 resulted in a recovery below 50 per cent for phenobarbital, therefore, extraction with a strongly alkaline solvent (0.1 M NaOH) was necessary as a further step.

Validation was carried out in accordance with ICH Guideline Q2(R1) using model suppositories of known compositions prepared as prescribed by the physician. After analysis of the preparation to be examined, amounts of theophylline, codeine and ephedrine were found to be greater than the acceptance limit. According to the 7$^{th}$ Edition of the Hungarian Pharmacopoiea and presuming a posology as stated on the prescription the level of codeine

slightly exceeded the maximum single dose allowed in case of a 1-year-old child and the amount of ephedrine might have exceeded the maximum daily dose. These results indicated that contribution of the poor quality of the extemporaneous preparation to the serious adverse reaction could not be excluded.

*Target screening of cathinone derivatives in unknown powder samples by HPLC-MS/MS; detection and identification of the new cathinone derivative 4-MEC*

Separation of the six known cathinone derivatives was accomplished within 8 min using gradient elution. An isocratic system was not feasible due to the relatively higher lipophilicity of MDPV. After examination of each MRM transition an LOD of approximately 2 pg was calculated for each substance which enables application of the method in areas other than pharmaceutical analysis (e.g. forensic chemistry).

Identification of 4-MEC was considerably facilitated by performing detailed fragmentation studies of the 6 other compounds. After HPLC-UV-MS/MS tests it was assumed that the unknown molecule was an analogue of mephedrone differing only by one excessive methylene group probably affecting the amino group. This was confirmed by the similarity of the UV spectra. Final structure elucidation was carried out by one- and two-dimensional $^{1}$H- and $^{13}$C-NMR analysis. Chemical structure of 4-MEC was clearly identified which was supported by high resolution MS data where $C_{12}H_{17}NO$ corresponding to the elemental composition of 4-MEC was calculated by the software as the only possible compound formula within the acceptable limits when allowed elements were restricted to carbon, hydrogen, oxygen and nitrogen. By carrying out group-identification reactions prescribed by Ph. Eur. it was also shown that the substance was present as hydrochloride salt. Tests also indicated that the powder sample was of high purity. To our knowledge, we were the first to present the IR spectrum of 4-MEC-hydrochloride in literature which may be used as a reference for identification purposes.

Finally, the HPLC-MS/MS method developed for targeted detection of 6 cathinone derivatives was successfully "extended" with respect to the new compound which appeared in the chromatogram between the peaks due to mephedrone and MDPV. Based on this experience it is assumed that the method may be applied to other emerging unknown cathinone derivatives which, however, should be previously identified by other orthogonal analytical techniques.

## Conclusions

The three new analytical methods described in the thesis substantially differ from each other from technical aspects as well as with respect to their purpose. However, each of them provides an equally relevant example of practical issues encountered by a drug control laboratory.

Market surveillance studies of approved medicinal products may involve multiple batches of preparations containing the same API but produced by different manufacturers. In this case both time and cost of analysis play an important role. Therefore, it is favourable to replace tests described in the documentation filed by the marketing authorisation holders often requiring different reagents and equipment by a common method. Since it is usually not feasible to check compliance with every single requirement of the specification, one or two characteristics are preferably chosen which may be indicative with respect to the aim of the given study.

The CE method developed by our laboratory fully meets these requirements. Quantification of API and the major degradation product in three different products was

carried out within one working day. These tests are stability indicating because, for example, monitoring degradation of the API may reveal unsatisfactory storage conditions or other problems affecting stability. Organic solvent consumption compared to HPLC is negligible which is beneficial from a financial point of view and also for the environment. In our opinion development and more frequent application of similar methods should be considered.

In contrast to approved medicinal products extemporaneous preparations are produced in small-scale and results of their analysis only apply to batches manufactured at the same time. Testing methods of individual preparations are rarely intended for routine purposes, therefore, in this case analysis time and costs are of minor importance. In the given example, the expert opinion concerning the extemporaneous suppository in question might have strongly influenced the question of responsibility of the pharmacist. This had to be kept in mind during development and validation of the method.

When general acceptance criteria for extemporaneous medicinal preparations were established technical aspects related to their production had to be taken into account. Thus, acceptance limits are less rigorous than in case of approved pharmaceuticals. Despite using this approach it was obvious that the levels of 3 out of 4 compounded active substances were higher than the maximum allowed dose. Nevertheless, classifying the sample as "non-compliant" does not necessarily imply any direct or indirect contribution to the described symptoms of intoxication. Therefore, in such cases particular care should be taken to include in the expert opinion only conclusions which can be unequivocally confirmed in the light of the results.

The case presented does certainly not question the need for extemporaneous medicinal products. However, it is emphasised that the quality required for approved products cannot be expected of a preparation manufactured in small-scale production in pharmacies. This underlines why it is important for physicians prescribing medicines of individual composition and for pharmacists working in accordance with the prescription to be aware of their responsibility. The lack of this awareness could entail that even an apparently minor mistake may have fatal consequences.

Drug control agencies often have to deal with partially unknown substances which in fact are not classified as pharmaceuticals. Illegal medicinal products belong to a category which leads to overlapping with areas like toxicology, forensic science or food chemistry. This is recognised as a new challenge from both the analytical and the strategical point of view. For such samples the concept of quality is essentially interpreted on a completely different level. Questions to be answered are usually restricted to identification of the relevant ingredients and, where justified, quantification of these components.

Besides eliminating the problem of often expensive and hardly available reference substances the HPLC-MS/MS target screening method presented in the thesis also provides the laboratory an opportunity to save time. For a sufficiently effective application, however, one should be aware of the limits of the method: it can only be used for detection and identification of the target group, and in itself it is not suitable for complete identification of newly emerging molecules with unknown chemical structures.

As the significance of pharma industry is growing, as quality specifications get more and more rigorous and detailed and as a result of frequently changing approaches related to constant improvement, from time to time, official drug control agencies come across new tasks. Application of modern instrumental analytical techniques seems to be the only chance to keep up with these emerging challenges. By presenting these three examples we also attempted to demonstrate that efficient and high quality drug control should be a common interest. We are therefore convinced that professional innovation and financial investment which are both indispensible to promote this field may be repaid already after a short period of time.

**Publications forming the basis of the PhD thesis:**

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