Ph.D. Thesis

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

Éva Kalmár, Pharm.D.

Szeged

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Abbreviations

ACN:	acetonitrile
AMFZ:	4-(dimethylamino)antipyrine
API:	active pharmaceutical ingredient or active substance
ASA:	acetylsalicylic acid
CMC:	critical micelle formation concentration
EP:	see Ph Eur
f:	displacement factor
HPLC:	high-performance liquid chromatography
MeOH:	methanol
NIR:	near infrared spectroscopy
NMR:	nuclear magnetic resonance
OTC:	over-the-counter
Ph Eur:	European Pharmacopoeia
RP-HPLC:	reversed-phase HPLC
R&D:	research and development
UHPLC:	ultra high-performance liquid chromatography
US:	ultrasonic
USP:	United States Pharmacopeia
UV/VIS:	ultraviolet/visible
W35TT:	adeps solidus compositus

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List of publications and lectures

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)

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Fast efficient and robust UHPLC determination of 4-dimethylaminoantipyrine from different types of suppository vehicles
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21-24. June 2012, Košice, Slovakia (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)

Other publications, lectures

Gyógyszeranalitika gyakorlati útmutató (fejezetek: komplexometria, konduktometria, HPLC analízis, atomspektroszkópia)
Gyakorlati jegyzet, SZTE GYTK, Gyógyszeranalitikai Intézet (book chapter)

K. Jósvay, A. Buhala, Z. Winter, T. Martinek, E. Wéber, L. Németh, A. Hetényi, **É. Kalmár**, G. Dombi, Z. Oláh, G. Szakonyi
TRPV1 and calmodulin interaction
EFIC® – 8th "Pain In Europe" Congress
9-12. October 2013, Firenze, Italy (poster)

G. Szakonyi, K. Jósvay, A. Buhala, Z. Winter, **É. Kalmár**, F. Ötvös, Cs. Vízler, G. Dombi, Z. Oláh
Investigation of vanilloid receptor – a target for novel pain killers
5th BBBB International Conference
26-28. September 2013, Athens, Grece (poster)

A. Buhala, K. Jósvay, Z. Winter, L. Pecze, **É. Kalmár**, Gy. Dombi, Z. Oláh, G. Szakonyi
Structural Analysis of the human TRPV1 receptor
Hungarian Molecular Life Sciences
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É. Kalmár
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Z. Winter, K. Jósvay, **É. Kalmár**, F. Ötvös, Z. Oláh, T. Letoha, G. Dombi, G. Szakonyi
A TRPV1 csatorna szerkezetének vizsgálata
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É. Kalmár, H. D. Szűcs, G. Dombi, G. Szakonyi
AcrB homológ membránfehérjék expressziója *Escherichia coli*ban
40. Membrán Transzport Konferencia
18-21. May 2010, Sümeg (poster)

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IX. Clauder Ottó Emlékverseny
23-24. April 2009, Budapest, HU (lecture)

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. Literature

2.1. Tested pharmaceutical dosage forms

2.1.1. Oral powders

An oral powder as a pharmaceutical dosage form containing solid, loose, dry ingredients, including one or more APIs with or without excipients. It is generally administered in or with water or another suitable liquid. It may also be swallowed directly. It is presented as single-dose or multidose preparations. Multidose oral powders require the provision of a measuring device capable of delivering the quantity prescribed. Each dose of a single-dose powder is enclosed in an individual container, for example a sachet or a vial [1].

Oral powders are currently very popular dosage forms. Especially favoured are the granule forms of various OTC preparations, such as ACC®, Aspirin® or Neo Citran®. Their main advantage over compressed dosage forms is the larger specific surface, the less significant incompatibility issues and the comparative ease of adding taste maskers and colouring agents during formulation. When a rapid effect is desired, the API, for example an analgesic drug can be applied in oral powder dosage form (Flector®).

2.1.2. Suppositories

Suppositories are currently very popular formulations especially in paediatrics, where they can be used for the effective lowering of fever. The choice of a suppository as the mode of drug delivery is justified in all cases when oral delivery is impossible, that is an unconscious or vomiting patient, or in the case of infants.

The therapeutic effect of correctly applied suppositories can be compared with that of injections because the API can penetrate from the lower tract of the rectum to the vena anales, thereby avoiding the vena portae and the liver, and can exert a systemic effect when transported to the vena cava inferior, so the API does not undergo a first pass effect (**Figure 1**).

The therapeutic use of suppositories has another aspect worldwide at present. The suppository dosage form is widely used for various therapeutic indications, making use of the feature that the local effect of the suppository can be transformed into therapeutic benefit (e.g. in the treatment of asthma, ulcerative colitis, ulcerative proctitis or colorectal cancer in paediatric practice) [2-8]. The treatment of acute malaria in children requires combination

therapy in order to avoid the development of multidrug resistance. In these scenarios, it is a plausible solution to deliver one of the drugs of the combination in a suppository [9, 10]. Thus, a rapid systemic effect can be achieved. For the delivery of several non-steroid anti-inflammatory drugs, such as paracetamol or indometacin, the efficacy of the suppository form is equivalent or superior to that of the oral route [11-14].

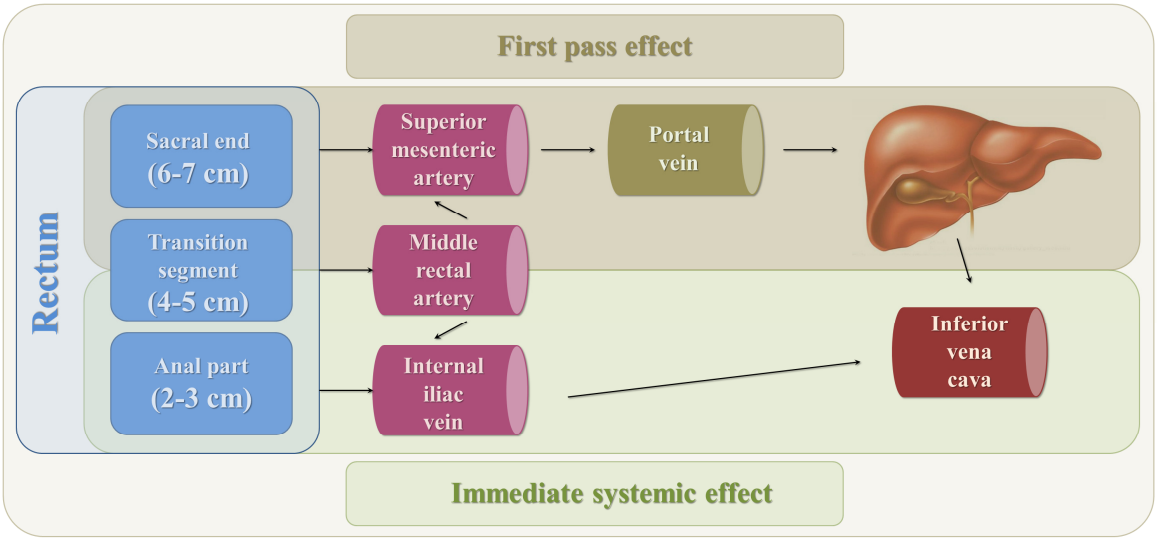


Figure 1 Potential uptake locations of the drug from the different sections of the rectum

In Hungarian pharmaceutical practice, extemporaneous products including suppositories are just as popular as factory-produced medicines. Extemporaneous products comprise part of personal therapy, and take into account the physical status, age and other diseases of the patient. Extemporaneously produced pharmaceuticals are used particularly in paediatric clinical departments.

Approximately 80% of the suppositories used in Central Europe are produced extemporaneously by moulding technique. In clinical pharmacies quantities of 100-300 and in independent pharmacies 10-12 suppositories are generally moulded as one batch. Suspension suppositories in particular are formulated with a solid fat vehicle (e.g. Witepsol 35) or a combination of this suppository base with surfactants [15]. The core of this technology is the dispersion of the finely powdered drug in the molten suppository base, after which the suspension is moulded under continuous stirring. The viscosity of fatty suppository bases is very low, and decreases still further with the increase of temperature, causing rapid sedimentation of the suspended particles and leading to an inhomogeneous product. When the liquid mass is moulded at around the solidification point, solidification occurs immediately when the mass enters the mould, making further additions of the base and drug impossible. In the calculation of the suppository base weight, Eq. 1 must be applied:

$$T_m = E - \sum_{i=1}^{n} f_i \cdot s_i \quad (1)$$

where T_m is the suppository base to be weighed, E is the calibration constant of the mould, f_i is the displacement factor of the ith component and s_i is the weight of the ith component. During the calculation of a correct formula, it is not sufficient to subtract the weight of the solid components from the final weight of the suppository to obtain the required amount of suppository base. We have to know the value of E for the specific mould and the specific suppository base, which can be determined through independent measurements. Ten suppositories are moulded with the mould, using the pure base, and after cooling they are weighed and the average suppository weight is calculated. This average value will be used as the calibration constant of the mould for the specific base. As the density of the API incorporated in the suppository can differ from that of the base, the displacement factor (f) is required to compensate the difference in densities. The value of f, which shows how much base will be displaced by unit weight of API, can be calculated from Eq. 2:

$$f = \frac{100 \cdot (E - G)}{G \cdot x} + 1 \quad (2)$$

where E is the weight of the blank suppository containing only base, G is the weight of the suppository containing the API in known concentration, and x is the API content of the suppository in weight percentage. If the pharmacist fails to carry out the steps in strict accordance with the rules, significant deviations will be observed in the results of the homogeneity of the batch and in the total drug content of the batch. In pharmacies, the f values of the most frequent APIs in the most common bases are not generally available. According to good manufacturing practice, pharmacists apply the principle of overage during the calculation of the batch composition, but an incorrect calculation for the amount of vehicle required and other technological errors may lead to serious deviations in the final dosage for the individual suppositories [16-18].

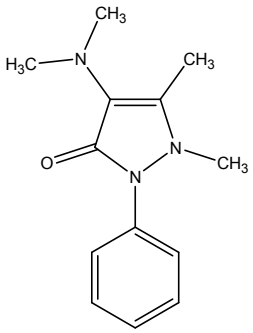
In Hungarian pharmaceutical practice, moulded suppositories are formulated predominantly with three suppository bases: adeps solidus, massa macrogoli and W35TT, which contains surfactants. The lipophilic adeps solidus is officially included in Ph Eur as hard fat or Witepsol W35. Massa macrogoli is a hydrophilic base, which contains: macrogol 1540 and Span 20. W35TT is a special lipohydrophilic base, which is included officially in FoNo. It is a mixture of 95 w/w% of hard fat, 2.5 w/w% of Tween 20 and 2.5 w/w% of

Tween 61. In consequence of procurement issues relating to Tween 61, Tween 60 is nowadays used instead.

Numerous studies that have focused on the liberation of drugs from suppositories containing surfactants from the aspect of pharmaceutical technology have clearly revealed that it is beneficial for a suppository base to have high hydroxyl group content. The usage of non-ionic surfactants is now suggested, but in lower amounts than those used in older recipes, which generally means lower than 3%, and preferably around 1% [19]. A high surfactant concentration may lead to the formation of micelles, which incorporate some of the API, impeding its release [20]. According to Ghorab et al. [21], the optimum amount of Tween 60 is 5%; higher proportions than that up to 10% had a lower effect on the release rate. Above 10%, the release rate is decreased due to micelle formation. Surfactants not only enhance release of the drug from the suppository base, but increase the permeability of the tissues surrounding the rectal lumen. Non-ionic surfactant Tween 20 showed outstanding effectiveness when used in 5% combined with lipophilic vehicles [22].

2.2. Analysed drug substances

2.2.1. Aminophenazone

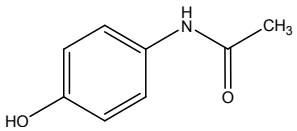


AMFZ is a phenazone derivative. It is a white crystalline powder which is soluble in water and freely soluble in alcohol. Its pK_a is 4.70, and its log P is 0.99. AMFZ is an antipyretic and analgesic drug, for example in Demalgon® tablet or Germicid® suppository. This API is frequently used in clinical paediatric practice in Hungary, especially as an extemporaneous dosage form [23-26].

The antifebrile effect of AMFZ develops especially quickly (comparable to that of injections) if the drug is taken rectally. An additional benefit is that its administration does not require specially trained staff. Agranulocytosis, one of the registered side-effects of the substance, has a very low incidence, while carcinogenicity, another possible side-effect, can be completely eliminated through rectal administration [27-35]. During its biotransformation, AMFZ is demethylated in two steps, catalysed by cytochrome P450 2B [28, 29]. The demethylated product then undergoes acetylation and is eliminated from the body as acetylaminoantipyrine. In the presence of nitrite ion at pH between 2.0 and 3.1, the carcinogenic nitrosamine derivative dimethylnitrosamine is formed in parallel with the demethylation. The physiological circumstances in the stomach provide a suitable medium for

this reaction to take place [36-38]. On the other hand, rectal administration of AMFZ completely eliminates the possibility of dimethylnitrosamine formation as the pH of the mucous fluid in that region is around 7.9.

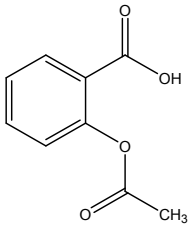
2.2.2. Paracetamol



Paracetamol or acetaminophen is one of the most frequently used antifebrile and painkiller drugs around the world. It has been used in Hungary only since 1990. It is incorporated in many well-known products (Rubophen®, Panadol®, Coldrex®, Mexalen®, Miralgin®, Neo Citran® and Saridon®). It is an aniline derivative. It is a white, crystalline powder, which is moderately soluble in water and freely soluble in alcohol. Its calculated pKa is 9.48 and its log P value is 0.53 [24, 26, 39].

Usually it is not classified as an NSAID because it does not show a significant anti-inflammatory effect. In the event of an overdose, it causes acute liver failure. This is due to the saturation of conjugation with sulphate and glucuronide systems, which generate nontoxic metabolites, leading to the conversion of paracetamol to the highly reactive intermediate metabolite N-acetyl-p-benzoquinoneimine (NAPQI) via the cytochrome P450 2E1 and 3A4 enzyme system, which becomes predominant. Excess amounts of NAPQI and glutathione are produced, which are responsible for decreased detoxification. Acetylcysteine can be used as the antidote of paracetamol toxication, which reduces paracetamol toxicity by rebuilding body stores of glutathione. Glutathione reacts with the toxic NAPQI metabolite so that it does not damage cells and can be safely excreted [40-42].

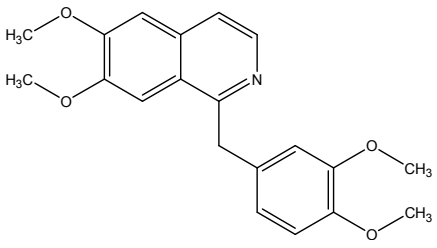
2.2.3. Acetylsalicylic acid



ASA is a white, odourless, crystalline powder, which is slightly soluble in water and freely soluble in alcohol. It is used as a painkiller, antifebrile or anti-rheumatic drug. Its pK_a is 3.83, and its log P is 1.25 [24, 26, 43].

For the mitigation of acute renal or gastrointestinal pain, the primary drug of choice is a NSAID such as ASA, paracetamol or ibuprofen [44].

2.2.4. Papaverine



Papaverine is a white, crystalline powder that is moderately soluble in water and alcohol. It is freely soluble in hot water. Its solubility can be increased by decreasing the pH of the aqueous medium. It is an alkaloid of opium. It has a smooth muscle relaxant effect [40, 45]. Its pK_a is 6.12 and its log P is 3.92 [26]. A number of drug products are available on the market for the treatment of smooth muscle spasm, e.g. in the biliary, renal and intestinal tracts (BILA-GIT®). Such conditions are frequently treated with combined products, which contain a smooth muscle antispasmodic together with one or more NSAID painkiller drugs [46]. The combination of papaverine-HCl or papaverine base and ibuprofen or indometacin is nowadays commonly used, especially for the treatment of dysmenorrhoea. As regards the administration of papaverine, the research focus has shifted in recent years from the gastrointestinal tract to the coronary arteries [47] and the therapy of an erectile dysfunction [48-50, 40]. Nevertheless, in pharmaceutical practice, papaverine is still commonly prescribed as an antispasmodic to relieve gastrointestinal and menstrual spasms.

2.3. Analytical methods

2.3.1. Development of HPLC assay

As an analytical technique, HPLC possesses a very impressive history, which has been extensively studied by many authors [51-55]. In my thesis, the focus is placed on the development of sample preparation methods and assay determination of pharmaceutical products. A deep theoretical introduction on the separation of small molecules will therefore not be included.

One of the most important tasks of a chromatographic analyst in pharmaceutical R&D is the development of analytical methods for the assay of pharmaceutical products and validation of the methods before submission.

The most challenging key step in this process, especially in the case of complex dosage forms (such as suppositories, extended release tablets, etc.), is the sample preparation. In this step, the API must be separated from the matrix, which can be a very complex task if both identification and quantitative determination are required. To achieve optimum recovery, the total API content of the product must be liberated. The European Pharmacopoeia does not

provide any guidance as concerns general sample preparation for the assay of any dosage form; details are included only in the pharmaceutical technological procedures. The US Pharmacopoeia, on the other hand, contains monographs about pharmaceutical products, including suppositories [16]. According to the literature, samples can be divided into 4 groups: solid or liquid pharmaceutical products, biotechnological samples (proteins) or biological samples (blood, urine, tissue fluid, etc.). In the case of solid dosage forms, dissolution of the API from the finely ground powder of the sample with an appropriate solvent is necessary. It should be noted that the solvent must be compatible (miscible) with the chosen mobile phase of the chromatographic system. In the event of liquid dosage forms, this step is much simpler. On the other hand, solubility problems may arise, partition of the API between solvents of different polarity can be observed, or a change in solubility can occur with the change of pH. It may be generally stated that physical interactions of APIs and excipients that potentially cause problems during recovery are studied very rarely. Sample preparation should be handled within the scope of method development.

During method development for HPLC, there are many options beyond those in the scientific literature to design or to predict the behaviour of the system. The main goal is always to separate the analytes from each other and from other components of the sample in a rapid, reproducible, accurate and robust method which provides optimum peak shape and theoretical plate number.

HPLC method development is currently aided by many expert systems, such as the Pallas program package or the Marvin program package. In order to design the separation, it is necessary to know the basic physico-chemical parameters (log P, pK_a and log D), which can provide guidance to reach the optimum method in the fewest development steps. log P determines the polarity of the compound, which is a fundamental parameter of the retention. pK_a facilitates determination of the pH range in which a robust method can be developed, and also aids the choice of appropriate buffer system. log D describes the change in polarity of the compound vs. pH. It is a good indicator of how the retention may change in the studied pH range. The log D vs. pH function presents the distribution change of the dissociated and non-dissociated forms. Thus, the mobile phase composition can be effectively designed on the basis of the predicted pK_a and log D vs. pH curve, obtained from the expert system. In some cases, the sequence of elution of the components can also be effectively estimated.

If the retention factors of the components differ too widely (k'_1-$k'_2 > 10$), it is suggested to apply gradient elution instead of isocratic elution. In gradient elution, the composition of the mobile phase is varied as a function of time. The application of gradients can effectively

decrease the retention of selected components if the concentration of the stronger component in the mobile phase is increased.

2.3.2. The CMC and its determination

One of the most characteristic properties of a surfactant is its CMC. In a specific solvent system, this is the concentration above which individual surfactant molecules spontaneously aggregate and form micelles through secondary interactions. Several methods are available for the determination of CMC. The most widely used techniques are the measurement of surface tension, conductance in the case of ionic surfactants, UV/VIS spectrophotometry, NIR spectroscopy, turbidimetry and densitometry [56, 57]. As the suppository base studied in the present work contained Tween 20 and Tween 60, in **Table 1** the CMCs of these materials determined with different methods in previous studies are listed.

Table 1 CMCs of Tween 20 and Tween 60

TWEEN 20					
Reference	Method	Temperature	Medium		
			H_2O	D_2O	Spec.[a]
[58]	Surface tension	RT	0.0804	-	-
[59]	NIR	RT	0.08	0.12	70
[60]	Surface tension	RT	0.0488	-	-
[61]	Densitometry	24.88 °C	0.097	-	-
[62]	Surface tension	RT	0.059	-	-
[63]	Spectrophotometry of iodine absorbance	RT	0.055	-	-
	Shift in λ_{max}	RT	0.0557	-	-
	Surface tension	RT	0.0488	-	-
[64]	Surface tension	RT	0.048	-	-
[65]	Dye micellization	RT	0.042	-	-
	Surface tension	RT	0.011	-	-
TWEEN 60					
[61]	Densitometry	24.88 °C	0.068	-	-
[63]	Spectrophotometry of iodine absorbance	RT	0.0209	-	-
	Shift in λ_{max}	RT	0.0196	-	-
	Surface tension	RT	0.0206	-	-
[64]	Surface tension	RT	0.021	-	-
[65]	Dye micellization	RT	0.022	-	-
	Surface tension	RT	0.0055	-	-

[a] 1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide $[EMIm]^+[Tf_2N]^-$.

When a colloidal solution is irradiated with visible light, the incident coherent beam is scattered. The intensity of the transmitted light is therefore lower than that of the incident

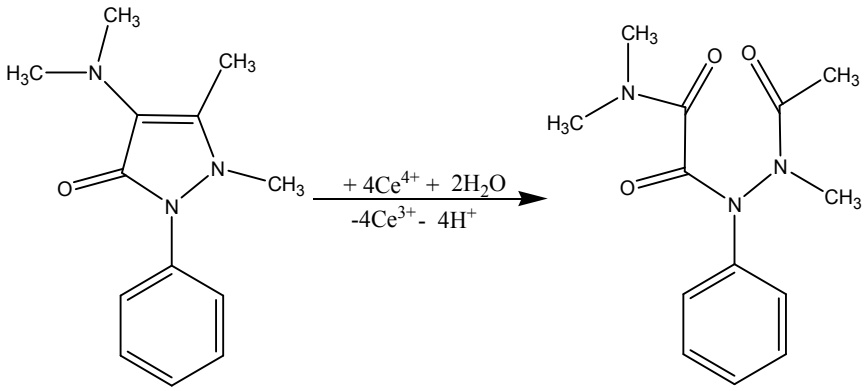
light, and scattered light can be detected in any direction around the incident beam. This phenomenon is called pseudoabsorbance or turbidity and is described by Eq. 3:

$$\tau = \frac{1}{l} \ln\left(\frac{I_0}{I_t}\right) \tag{3}$$

where τ is the turbidity, I_t is the intensity of the transmitted light, I_o is the intensity of the incident light and l is the path length. As the light absorption properties of the colloidal solution before and after micelle formation differ, the rate of turbidity increase changes when the surfactant concentration is increased. This method therefore appeared appropriate for CMC determination. The intensity of the scattered light in connection with the turbidity of the solution is influenced by the size of the scattering particles, the difference between the refractive indices of the particles, the medium (contrast) and the interaction of the particles. In dilute solutions, interactions between particles can be neglected, and thus the increase in turbidity can be ascribed to the aggregation of the particles or in other words the formation of micelles [66].

2.3.3. Cerimetric titration of AMFZ

The basis of the determination is a cerimetric redox titration method [67], during which the nascent oxygen evolved from the reaction of Ce(IV) with water oxidizes AMFZ. The end-point of the titration is observed by the change in colour of ferroin present as indicator.



AMFZ Dioxypyramidone

The Ce(IV) ion oxidizes the water according to the following equations :

$$Ce^{4+} + H_2O^{\bullet\bullet} \rightarrow Ce^{3+} + [H_2O^{\bullet}]^{+}$$

$$[H_2O^{\bullet}]^{+} \rightarrow HO^{\bullet} + H^{+}$$

$$2\ HO^{\bullet} \rightarrow H_2O + O^{\bullet\bullet}$$

The produced nascent oxygen ($O^{\bullet\bullet}$) oxidizes the pyrazolone ring to dioxypyramidone [68, 69].

3. Materials and methods

3.1. Materials

The following materials were used in these studies: AMFZ (Sigma-Aldrich, St. Louis, MO, USA), paracetamol (Ph Eur 6.0, Phoenix Pharma Zrt., Hungary, Lot. No.: 1011204), papaverine-HCl (Molekula, Shaftesbury, UK), ASA (Ph Eur 6.0, University Pharmacy, University of Szeged, Szeged, Hungary), MeOH (Chromasolv for HPLC, Sigma-Aldrich, St. Louis, MO, USA), ACN (VWR, Prolabo, Fontenay-Sous-Bois, France), sodium acetate (Reanal, Budapest, Hungary), sulfuric acid 96% (Analyticals Carlo Erba, Milano, Italy), acetic acid 96% (VWR, Prolabo, Fontenay-sous-Bois, France), sodium hydroxide (Reanal, Budapest, Hungary), sodium chloride (VWR, Prolabo, Leuven, Belgium), potassium dihydrogenphosphate (Spektrum 3D, Debrecen, Hungary) and potassium hydroxide (Reanal, Budapest, Hungary), Suppositorium antipyreticum pro parvulo FoNo VII. (Naturland Ltd., Hungary, Lot. No.: 1938-1112 and Parma Produkt Ltd., Hungary, Lot. No.: 1209-1106). Throughout the experiments, HPLC grade solvents were used. The solvents and the aqueous solutions were prepared with triple distilled water. During the spectrophotometric measurements, MeOH (VWR, Prolabo, Fontenay-sous-Bois, France), TWEEN® 20 (Sigma-Aldrich, St. Louis, MO, USA) and TWEEN® 60 (Sigma-Aldrich, St. Louis, MO, USA) were used.

The suppository bases applied were hard fat and W35TT (University Pharmacy, University of Szeged, Szeged, Hungary).

The divided powder samples in 4.1. Part I contained approximately 17.0 mg paracetamol, 26.0 mg ASA and 5.0 mg papaverine in a homogeneous mixture. For the stock solution, 48.0 mg powder was weighed with analytical precision into a 50.0 ml volumetric flask, dissolved and made up to volume with the solvent, phosphate buffer (25 mM, pH 3.43) : ACN (85:15, V/V). During the preparation, the sample was heated to 40 °C, this step being required for the complete dissolution of ASA, which has low solubility (slightly soluble according to Ph Eur) in water. For the working sample solution, 3.0 ml stock solution was diluted to 10.0 ml and filtered through a 0.45 μm Millipore syringe filter before injection.

Volumetric solutions for the cerimetric titrations in 4.3. Part III were prepared with the following materials: cerium(IV) sulfate tetrahydrate (Panreac, Barcelona, Spain), sulfuric acid 96% (Farmitalia Carlo Erba, Milano, Italy) and ferroin-solution, 1/40 M (Reanal, Budapest, Hungary).

Factory-made suppositories were used during the comparison of the analytical methods. The reference product was Suppositorium antipyreticum pro parvulo FoNo VII. Naturland (Naturland Magyarország Kft., Budapest, Hungary), which contained 150 mg AMFZ per suppository in solid fat suppository base. One box contained six suppositories [70].

The studied samples in 4.3 Part III were prepared in regular pharmacies by a moulding technique, according to the following prescription. Ten suppositories were prescribed with a labelled claim of 100 mg AMFZ in each suppository. The choice of vehicle for the suppository was left to the responsibility of the pharmacist. Practically all of the samples were prepared with solid fat. In each case, predetermined technological errors (known to us) were made during the manufacturing samples.

3.2. Methods

3.2.1. Preparation of reference solutions and establishment of system suitability

The API contents of the samples were quantified by reference to reference solution in the appropriate solvent mixtures with concentrations presented in **Table 2**, which corresponded to the theoretical 100% concentration level of the sample solutions to be examined. Two reference solutions were prepared from independent stock solutions in order to check the system suitability by the following procedure.

Table 2 Concentrations of standard APIs

	Concentration (mg/ml)	Solvent
Divided powder		
Paracetamol	0.100	Phosphate buffer (25 mM, pH 3.43) : ACN (85:15, V/V)
ASA	0.150	
Papaverine	0.030	
Suppository		
AMFZ	0.075	MeOH: water (50:50, V/V)
Paracetamol	0.075	MeOH : ACN : 50 mM phosphate buffer (pH 6.3), (50:5:45, V/V/V)

The precision of the injections was checked before all measurement sets by injecting the first reference solution 5 times. The system was considered suitable if the RSD% of the 5 replicate injections did not exceed 2.0%. The accuracy of the calibration was checked by injecting the second reference solution twice. The results were accepted if the correlation factor (see Eq. 4) calculated from the average response ratio of the two reference solutions was not more than 2.0.

$$\text{Correlation factor} = \left| 1 - \frac{A_{Std1} \cdot w_{Std2}}{A_{Std2} \cdot w_{Std1}} \right| \cdot 100\% \quad (4)$$

where A_{Std1} and A_{Std2} are the average peak areas of the replicate reference injections, while w_{Std1} and w_{Std2} are the weights of the reference substances used to prepare the solutions.

The symmetry factor of the main peak of interest was also monitored throughout the measurements; it had to be between 0.7 and 2.0 for the analysis to be started.

3.2.2. Titrimetric analysis of suppositories with AMFZ

During the sample preparation, 1 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.

3.3. Instruments and other equipment

For mobile phase degassing and sample sonication a DLS 310-T DONAU-LAB-SONIC US bath was used.

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 [26].

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.

4. Results

4.1. Part I. Development and validation of HPLC assays

4.1.1. Chromatographic separation problems of drugs with different polarities

Many authors have described the simultaneous determination of paracetamol and ASA in various pharmaceutical dosage forms and also in blood or urine samples [71–81], but the available literature on the HPLC analysis of papaverine is quite limited. Mostly, the presence of papaverine together with opiates has been studied [82–90] and many findings are available as concerns its identification in blood samples from opiate drug users [83, 86, 87, 89, 90]. It is very rarely detected by means of UV/VIS photometry in chromatographic methods.

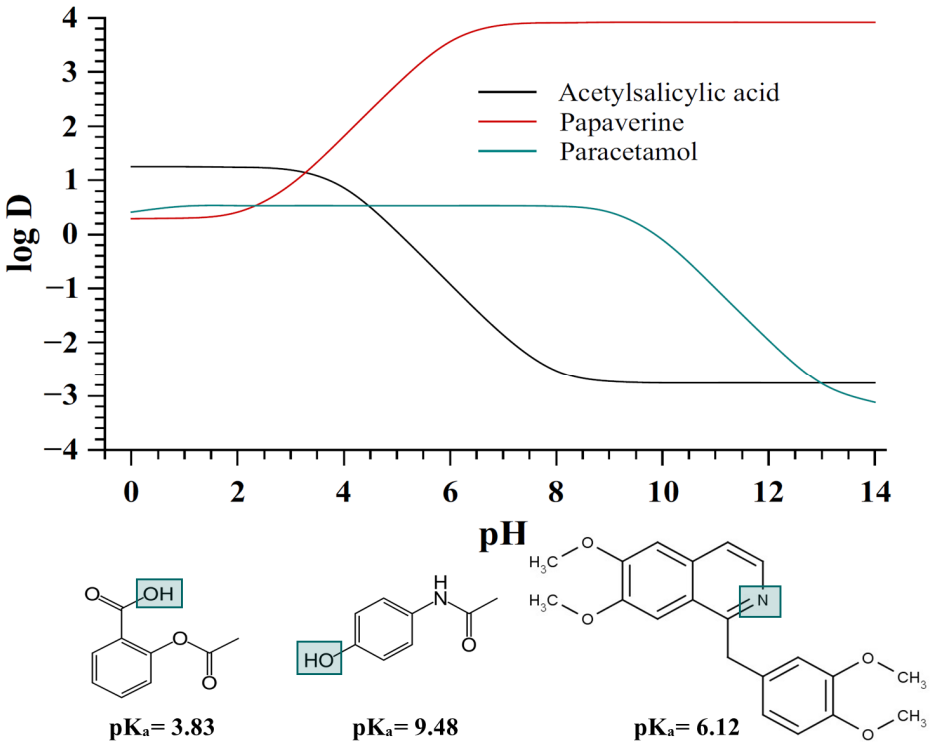


Figure 2 log D vs. pH curves of paracetamol, ASA and papaverine

4.1.1.1. Method development strategy

As the first step of chromatographic method development, the chemical properties of the drugs, which may influence the separation, were determined. Particularly the separation of papaverine and ASA can be difficult to achieve, in view of the specific pK_a values and the

log D vs. pH curves (**Figure 2**). The pH of the applied aqueous mobile phase was one of the key parameters that affected the separation. The range between 2 and 8 was optimum from the aspect of the stationary phase, but the range between 1 and 6 was not appropriate for the separation of papaverine, which contains 1 basic nitrogen with a pK_a in the upper part of the range. The ratio of dissociated and undissociated forms of ASA changes in the pH range 3-8. At pH > 6 (which is beneficial for papaverine separation), ASA peak splitting was observed [26].

In light of the above findings, the most challenging task was to find the most appropriate combination of the boundary conditions, where the overall negative influence on the separation and elution of the analytes was least. pH 3.4 ± 0.05 was found to be a reasonable compromise for the pH of the aqueous phase. An assay of papaverine alone was reported in the application database of Agilent, which involved a similar pH in the aqueous mobile phase [91]. In this method, the aqueous eluent contained 25 mM potassium dihydrogenphosphate, but sulfuric acid was used to adjust the pH so as not to increase the phosphate concentration.

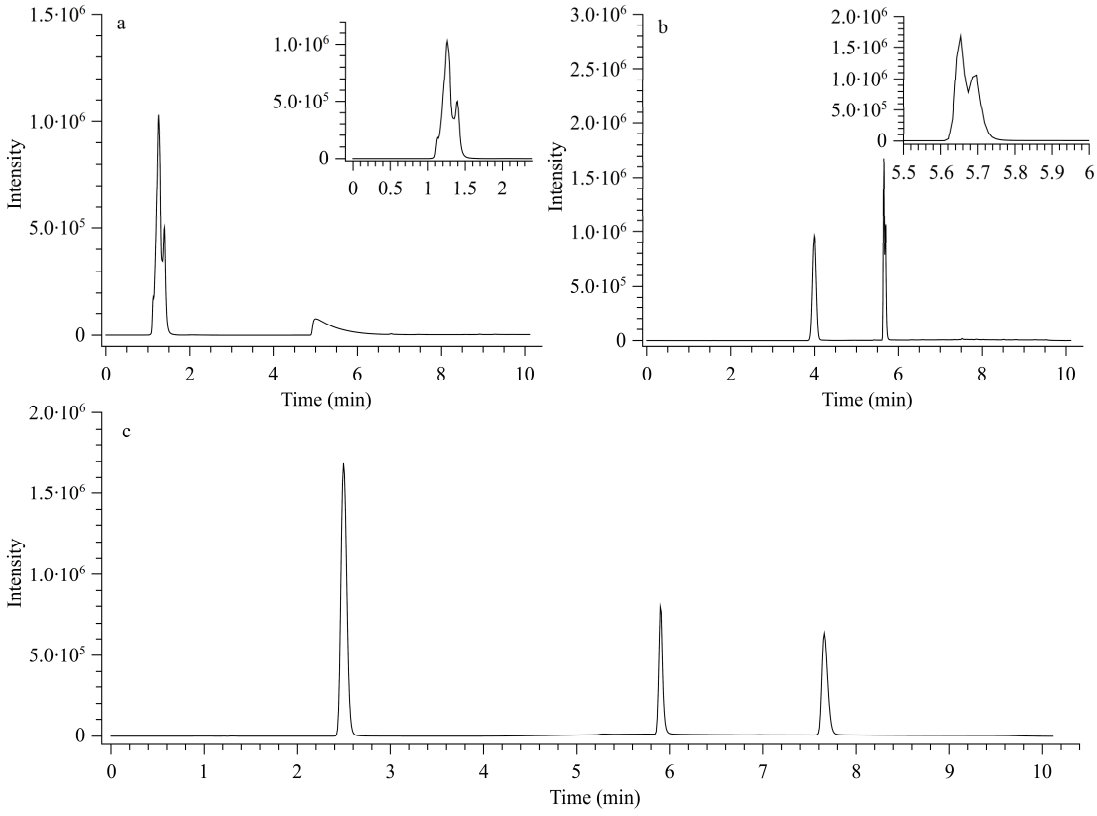


Figure 3 Chromatograms obtained on Hypersil ODS (a), Luna C18 (b) and Zorbax SB-18 (c) columns. Coeluting peaks are magnified in the insets

It can be seen in **Figure 2** that at pH 3.4 paracetamol and most of the ASA are in an undissociated form. The basic papaverine is at the beginning of the transient section of the equilibrium, which can be observed between pH 3 and 6 in the log D curve. The ratio of the organic modifier of the mobile phase, ACN, was linearly increased from 7% to 80% during the initial 16 min of the run time, and was then kept constant for 4 min. Between 20 and 22 min, the ratio of the organic modifier was linearly decreased to the initial level, at which it was held constant during the remainder of the run, to 25 min. A 1:1 (V/V) mixture of MeOH and the mobile phase was suggested as solvent in the literature method. The flow rate of the mobile phase was 1.5 ml/min and the separation was achieved on a Hypersil ODS column at 60 °C. The results of the runs under the above-described conditions can be seen in chromatogram (a) in **Figure 3**, where paracetamol and ASA were co-eluted. An initial isocratic hold was therefore inserted into the method before the gradient for the resolution of the co-elution, because the lower organic content selectively increased the retention times of the peaks, removing them from the void. In the new method, a constant 7% ACN section was applied during the initial 2 min, followed by a similar gradient as described above. At this point it became obvious that the hydrophobicity of the stationary phase was too low and the retention of basic papaverine was too high, so that it could not be eluted with acceptable peak shape within reasonable time, although the separation of the paracetamol and the ASA was ideal.

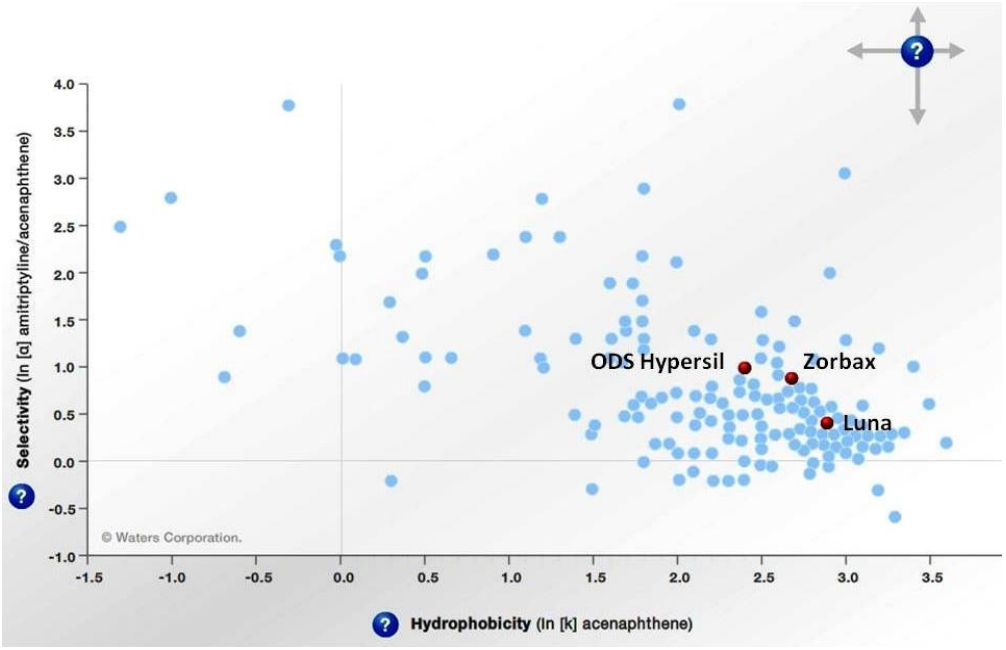


Figure 4 Selectivity and hydrophobicity comparison of the three columns in the database of Waters

For optimization of the peak shape, an alternative column had to be used. Two columns with different selectivity and higher hydrophobicity than that of the Hypersil ODS column were chosen on the basis of the data to be found in the comparative column selectivity database of Waters [92] (**Figure 4**), the Luna C18(2) and the Zorbax SB-C18 stationary phases. It is clear from chromatogram (b) in **Figure 3** that a hydrophobicity increase of less than one order of magnitude led to the successful elution of papaverine. This latter method resulted in the co-elution of ASA and papaverine on both columns. In order to resolve the peaks, the ACN content at the end of the gradient and in the second isocratic section had to be decreased from 80% to 25%.

This modification resulted in suitable separation for all three analytes on both Luna C18(2) and Zorbax SB-C18. ASA and the papaverine were eluted with higher resolution on the more selective Zorbax SB-C18 column. The retention parameters of the separated peaks on the three different columns are presented in **Table 3**. It is clear that the Hypersil ODS column was not suitable for the simultaneous separation of the three components, whereas the Luna C18 and Zorbax SB-C18 columns were equally appropriate; nevertheless, the results obtained on the Zorbax SB-C18 column were superior to those on the Luna C18 stationary phase as concerns its higher selectivity. Sample chromatograms measured on the three columns are presented in **Figure 5**.

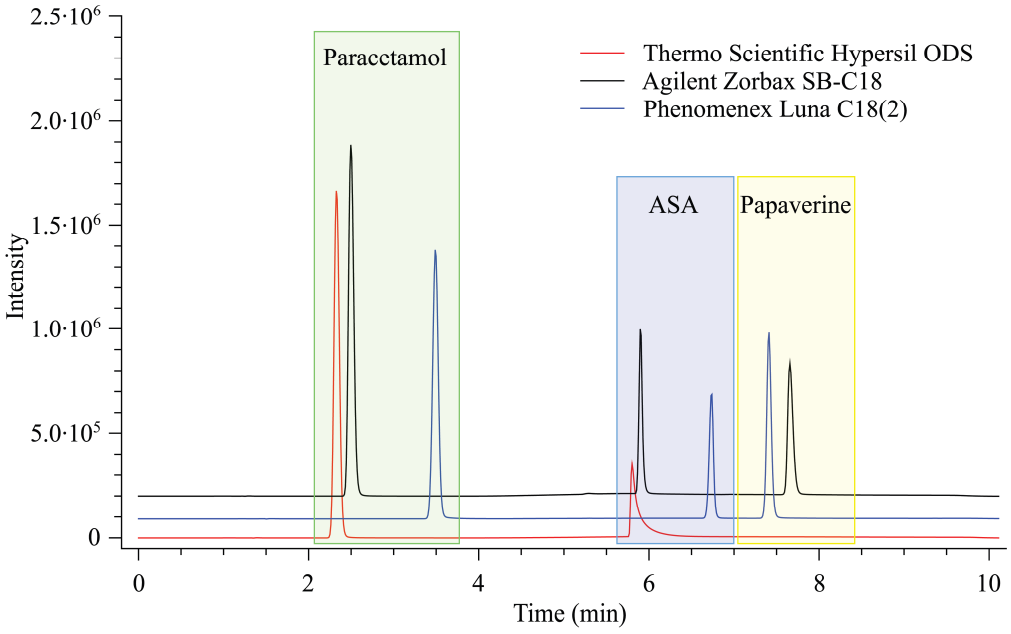


Figure 5 Comparison of the three stationary phases. It can be observed that papaverine was completely retained on Hypersil ODS

Table 3 Chromatographic parameters of the sample peaks on the three columns; k' is the retention factor, α the separation factor, R_s the resolution and t_R the retention time

Column type	Parameter	Paracetamol	ASA	Papaverine
Hypersil ODS	***k'***	0.549	2.866	-
	α	0.000	5.224	-
	R	-	25.511	-
	t_R	2.323	5.799	-
Luna C18	***k'***	1.324	3.487	3.937
	α	0.000	2.634	1.129
	R	-	29.949	7.039
	t_R	3.486	6.731	7.406
Zorbax SB-C18	***k'***	0.662	2.932	4.102
	α	0.000	4.432	1.399
	R	-	30.844	16.192
	t_R	2.492	5.897	7.653

4.1.1.2. The developed method

The mobile phase during the quantitative determination was a potassium dihydrogenphosphate (25 mM, pH 3.43) : ACN mixture. The details of the solvent gradient are to be seen in **Table 4**. The buffer was prepared with potassium dihydrogenphosphate, and the pH of the solution was adjusted to the desired value with 1 M sulfuric acid solution. The flow rate was 1.5 ml/min, the run time was 10 min and the column temperature was 60 °C. The chromatograms were recorded at 240 nm, at which wavelength all three components can be detected reproducibly. The choice of the detection wavelength was limited by the molar absorptivity of ASA, which is about one order of magnitude lower than those of the other components [93]. Although ASA is the main component of the mixture, its peak intensity is lower than that of paracetamol. During runs, the UV spectra (200-300 nm) of the components were collected for identification of the drugs. The column applied during method validation was the Zorbax SB-C18 150x4.6 mm, 3.5 μm column.

Table 4 Solvent gradient in the chromatographic method described in section 3.1

Time	ACN %
0.00	**7**
2.00	**7**
4.00	**25**
8.00	**25**
8.10	**7**
10.0	**7**

4.1.1.3. Validation

A full validation of the method according to ICH guideline Q2 (R1) [94] is presented here. The performance characteristics linearity, repeatability, intermediate precision, accuracy, specificity and robustness were tested. As the method was to be utilized for the rapid quality control of dosage units, which does not require the method to be stability-indicating, forced degradation studies were not conducted [95].

Linearity

The linearity of the method was examined in the concentration range between 0.02 and 0.04 mg/ml in the case of paracetamol, between 0.03 and 0.065 mg/ml for ASA and between 0.006 and 0.013 mg/ml for papaverine, these data corresponding to 70-130% of the nominal contents of the dosage units. The range was covered by use of six solutions, each diluted from two individually prepared reference solutions, so that the sequence of the stock solutions used for the dilutions alternated. The peak areas determined with LCSolution were plotted versus the concentrations of the solutions and a straight line was fitted to the points. The slope of the paracetamol fitted straight line was found to be $2.0171 \cdot 10^8$, the intercept was $1.5172 \cdot 10^3$ and R^2 was 0.9995. The slope of the fitted straight line in the case of ASA was found to be $4.9169 \cdot 10^7$, the intercept was $4.9344 \cdot 10^4$ and R^2 was 0.9997. Finally, the slope of the fitted straight line for papaverine was found to be $3.1811 \cdot 10^8$, the intercept was $-3.6861 \cdot 10^4$ and R^2 was 0.9997. This demonstrated that in the studied concentration range the response of the method was linear.

Precision/ Repeatability

Repeatability was checked on six individual samples according to the method described in section 3.1. For paracetamol and ASA, RSD% proved to be 0.4% and 0.6%, respectively, both of which are acceptable. The papavarine results gave the highest RSD%, 1.4%, but this is also acceptable when the very low nominal amount of drug in the sample is taken into consideration.

Precision/ Intermediate precision

The same analysis procedure was carried out by a different analyst on a different day, using a freshly prepared mobile phase. The results for the paracetamol component were an RSD% of 0.7% and a relative difference of 1.3% between the averages of the repeatability (Day 1) and intermediate precision (Day 2) results compared to the mean of the average

values measured for each. Both results can be accepted according to the principles of general pharmaceutical analytical practice. For the ASA, the RSD% of the individual results was 0.9%, while the relative difference between the repeatability and intermediate precision was 1.2%. For papaverine, the RSD% proved to be 2.1% and the relative difference of the mean values on the two days was also 2.1%. All three results are in accordance with the appropriate guidelines, and were therefore accepted.

Accuracy

The accuracy of the method was studied in the range between 70% and 130% of the nominal content of the powder. The results are shown in **Table S-1**. Although all of the average values fell between 95% and 105%, it should be mentioned that in the cases of ASA and papaverine most of the averages were below 100%, while in the case of paracetamol they were above 100%. This may raise a warning flag, but no trend was observed within the results that could be correlated with the increasing concentration of the sample groups.

Specificity

When the procedure was carried out with solvent as blank (the sample contained the API without excipients), no peak was detected at the retention times of the drugs.

Robustness

Examinations were made of the effects of changing the organic : aqueous ratio in the isocratic phases of the gradient, the pH of the aqueous phase, the flow rate of the mobile phase and the temperature of the column on the retention time and on the shapes of the analyte peaks. The results of the robustness studies (**Table S-2, Figure 6**) demonstrate that the ratio of the aqueous and organic phases exerted a great influence on both the retention time and the peak symmetry of the analyte. Variation of the pH of the aqueous phase caused only minor shifts in the retention times of the paracetamol and ASA peaks. The elution of paracetamol was not influenced by this parameter at all. In the cases of ASA and papaverine, the shift of the retention time in the opposite direction with the increase of pH caused an increase in resolution, which is in agreement with the increasing polarity of the components with pH. The flow rate change caused a minimal change in the retention time, proportional to the extent of the change. Flow rate changes did not influence the peak shape or plate numbers. Changes in column temperature did not cause significant changes in the retention times.

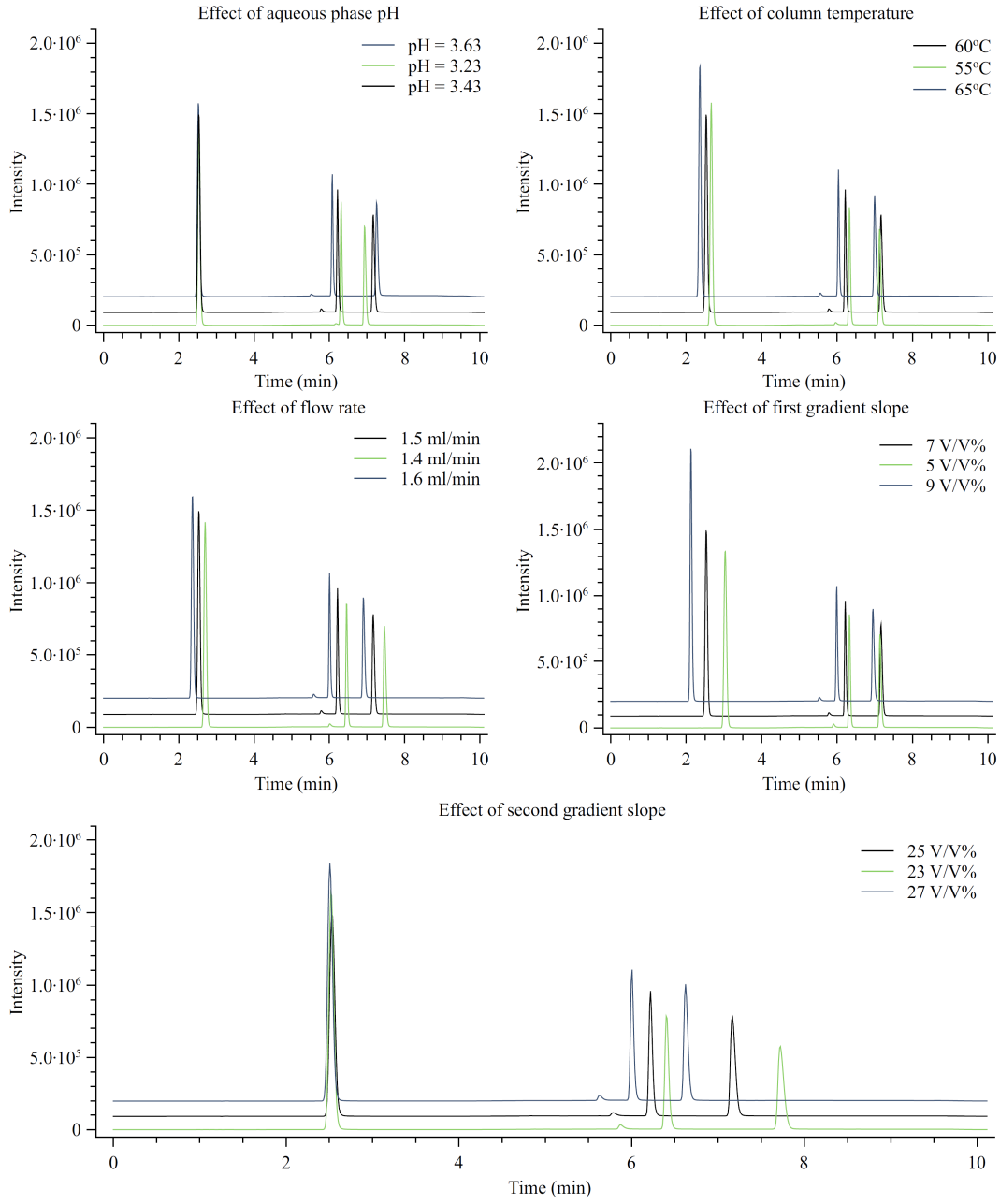


Figure 6 Robustness test results

Nevertheless, it is noteworthy that the retention of papaverine decreased with the decrease of temperature. Finally, variation of the organic : aqueous ratio, both at the start and at the end of the gradient, caused considerable changes in the peak retention times. Decrease of the organic modifier content of the initial hold increased the retention of paracetamol, while increase of the organic component pushed the peak very close to the void peak. Decrease of the organic modifier content at the end of the gradient increased the retention of both ASA and papaverine, this being more significant in the case of papaverine. On the other

hand, the papaverine peak shape became more asymmetric and the number of theoretical plates also decreased in this case. A change in the opposite direction led to decreases in the retention time of ASA and papaverine, the greater effect being observed for papaverine, and in this case the two peaks eluted too close to each other. This last change did not influence the retention of paracetamol; only a slight increase in the theoretical plate number was observed. The results reveal that the method is robust, and the peaks are well separated and elute with acceptable symmetry within the studied boundaries of the parameters.

4.1.2. Chromatographic assay of AMFZ and paracetamol for suppository study

Due to the complex nature of suppository matrices, a fast and efficient HPLC assay method was required to control the development of sample preparation. In the following subsections, the results of the development is presented.

4.1.2.1. Method development for AMFZ

A current, rapid, effective and state-of-the-art reversed-phase chromatographic method for instrumental routine analysis of suppositories containing AMFZ was to be set up. The literature search revealed that methods for the HPLC analysis of AMFZ were very rare and those found related to very low concentrations in biological fluids or tissues. On the other hand, many hits were found for the HPLC analysis of the pyrazolone derivative metamizole in tablet formulations, which could shed light on the initial steps of method development for AMFZ [96-103].

The stationary phase was chosen on the basis of the work of El Seikh et al. [96], but the initial scouting experiments revealed that the composition and the pH of the mobile phase had to be changed considerably. With MeOH–acetic acid (pH 2.78; 1.0%) (70:30, V/V) as mobile phase, the AMFZ peak eluted between 15 and 30 min and showed significant asymmetry (**Figure 7**). It was obvious that the mobile phase composition described by El-Seikh et al. would have given a much longer retention time. Simulations carried out with the Pallas software [26] showed that the pH of the aqueous part of the mobile phase should be > 4.5 to achieve acceptable robustness and peak shape (**Figure 8**).

A set of experiments was therefore designed using MeOH–sodium acetate buffer (pH 4.5 or 5.0; 0.05 M) (50:50 or 60:40, V/V) as mobile phase in various combinations. The shape of the AMFZ peak in the resulting chromatograms improved on increase of both the pH and the proportion of MeOH.

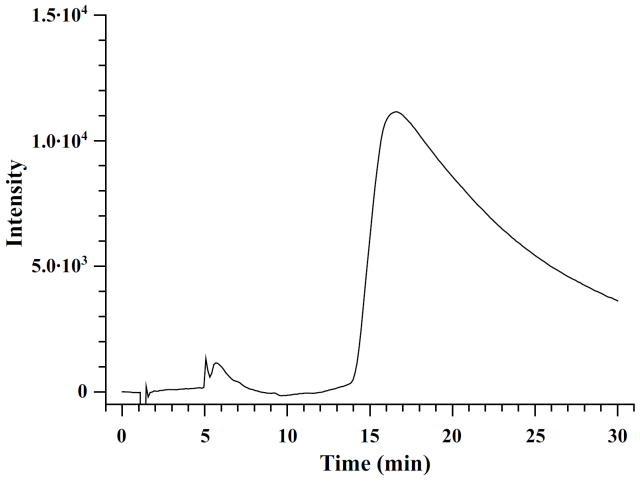


Figure 7 Initial chromatogram of development

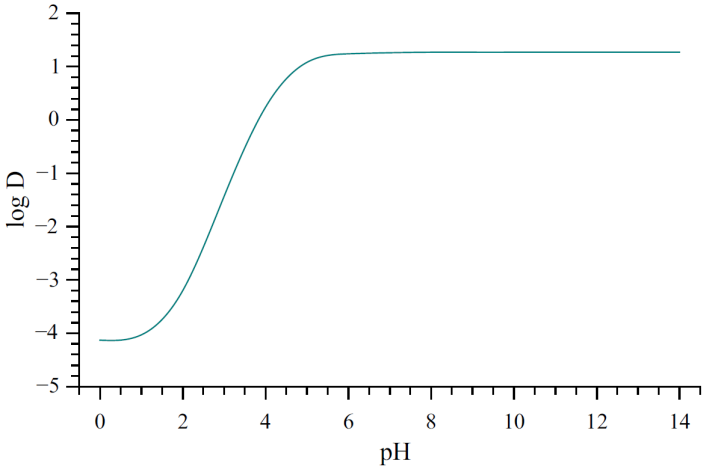


Figure 8 log D curve of aminophenazone by Pallas

In the final experiment, with MeOH–sodium acetate buffer (pH 5.5; 0.05 M) (60:40, V/V) as eluent, the symmetry factor of the AMFZ peak proved to be 1.43, and the peak width measured at the baseline was 0.2 min. It still seemed plausible to use acetate buffer at pH 5.5, where it has a somewhat lower buffer capacity, but the chosen concentration of 0.05 M compensated this.

4.1.2.2. Final assay for AMFZ analysis

The mobile phase was MeOH–sodium acetate (pH5.5; 0.05 M) (60:40, V/V). The pH of the sodium acetate buffer solution was set to 5.5 with acetic acid. The flow rate of the reversed-phase isocratic eluent was 1.5 ml/min and the run time was 5 min. The

chromatographic column was thermostated at 30 °C. The chromatograms were recorded at 243 nm. The retention time of aminophenazone was 1.8 min.

4.1.2.3. Development of a HPLC method for paracetamol assay

The method development was based on the parameters described in the literature. Phosphate buffer was prepared by mixing aqueous 0.05 M phosphoric acid solution with 0.2 M sodium hydroxide solution to reach pH 6.3 [77].

The isocratic mobile phase applied was a mixture of phosphate buffer (pH 6.3) and ACN (90:10) (V/V), filtered and degassed. The separation of the API was originally achieved on a Hypurity Advance column (150 x 4.6 mm, 5 µm, Thermo-Hypersil Keystone, Bellefonte, PA, USA, with a polar amide group embedded within a C8 chain). The flow rate was 1 ml/min, and the injection volume was 20 µl. The detection wavelength was set at 220 nm. The sample to be separated contained paracetamol and tramadol hydrochloride as APIs. The peak features of paracetamol were a retention time of 3.65 and a selectivity (α) of 2.50.

In the developed method, four parameters were refined. The isocratic elution remained, but the preparation of the aqueous phase was modified. The new buffer was prepared from 50 mM potassium dihydrogenphosphate, with the pH set to 6.3 ± 0.05 with 5 M potassium hydroxide solution. The final aqueous : organic ratio remained at 90 : 10, with ACN as the organic modifier. The application of potassium dihydrogenphosphate was necessary, because appropriate HPLC grade phosphoric acid was not available on stock.

The next modification was the change of the stationary phase. In the original method, the authors used a C8 column with an embedded polar group, but this was needed only for the separation of the other component (tramadol), and not for the retention of paracetamol. A general C18 column, Thermo Scientific Hypersil ODS, 150 x 4.6 mm, 5 µm, was therefore chosen. Furthermore, the flow rate was increased from 1 ml/min to 1.5 ml/min, the retention time of the paracetamol then decreasing to 2.4 min. The shorter running time (5 min instead of the original 8 min) was more plausible because the tested samples were monocomponent, and more injections could be completed within a given time in the absence of an autosampler. The detection wavelength was set to 241 nm because paracetamol has an absorption maximum at this wavelength (**Figure 9**).

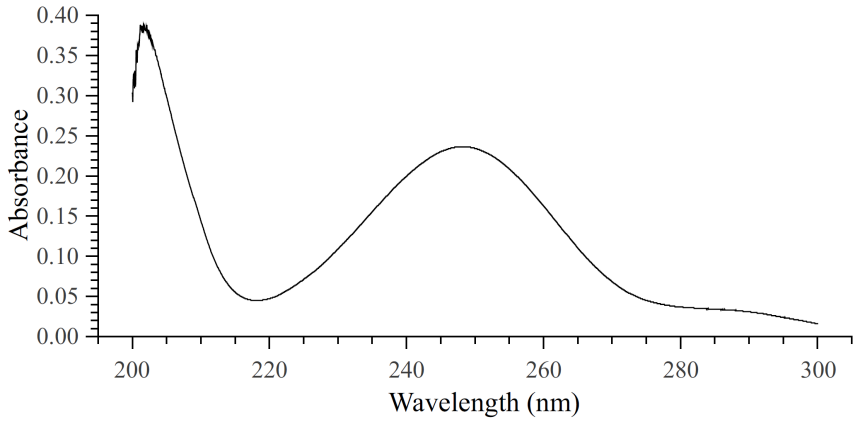


Figure 9 UV spectrum of paracetamol in MeOH

4.1.2.4. Final assay for paracetamol analysis

The mobile phase during the quantitative determination of paracetamol was ACN–potassium dihydrogenphosphate (pH 6.3; 0.05 M) (10:90, V/V). The buffer was prepared with potassium dihydrogenphosphate and the pH of the solution was adjusted to 6.3 with 1 M potassium hydroxide solution. The flow rate, the run time and the column temperature were the same as described in subsection 4.1.2.2. The chromatograms were recorded at 241 nm. The retention time of paracetamol was 2.3 min.

4.1.2.5. Validation

Full validation of both methods described in subsections 4.1.2.2 and 4.1.2.4 according to ICH guideline Q2 (R1) [94] has been carried out. The following performance characteristics have been studied: linearity, repeatability, intermediate precision, accuracy, specificity and robustness. As the methods were to be used for the rapid quality control of dosage units, which did not require the method to be stability-indicating, forced degradation studies were not conducted [95].

The repeatability, intermediate precision, accuracy and specificity studies were carried out with three vehicles in the case of AMFZ and with W35TT in the case of paracetamol. These vehicle specific results are shown in section 4.2.6.

Linearity

AMFZ method

The linearity of the method was examined in the concentration range between 0.025 and 0.150 mg/ml, which corresponds to 50-450% of the nominal content of the suppositories. The

higher limit was chosen with regard to the fact that initial experiments gave individual results in this concentration range. Thus, it was necessary to check the method at extremely high active substance concentrations. The range was covered by 7 solutions each diluted from 2 individually prepared reference solutions so that the sequence of the stock solutions used for the dilutions alternated. The peak areas determined with LCSolution were plotted versus the concentration of the solutions and a straight line was fitted to the points. The slope of the fitted straight line was found to be $3.498 \cdot 10^7$, the intercept was $-5.165 \cdot 10^4$ and R^2 was 0.9998. This proved that in the proposed concentration range the method was linear.

Paracetamol method

The linarity of the method was tested analogously to AMFZ. Linearity samples were prepared from 2 standard solutions, 0.5 mg/ml (A) and 0.6 mg/ml (B) paracetamol in MeOH. 7 solutions were prepared, which corresponded to 50-300% of the nominal content of the suppositories and using both individually prepared stock solutions alternatively. The target concentration was 0.05 mg/ml. The slope of the fitted straight line was found to be $6.210 \cdot 10^7$, the intercept was $7.474 \cdot 10^4$ and the goodness of fit, expressed by R^2, was 0.9963. This proved that in the proposed concentration range the method was linear.

Stability of standard and sample solutions

Table 5 Results of solution stability studies

Time/hours	Standard solution		Sample solution	
	Area	Relative difference %	Area	Relative difference %
0	2903843	-	2076275	-
18	2909154	0.2	2075720	0.0
24	2904895	0.0	2073721	-0.1
39	2902092	-0.1	2074466	-0.1
48	2904450	0.0	2073840	-0.1
63	2906272	0.1	2074709	-0.1
72	2903753	0.0	2080587	0.2
96	2904386	0.0	2077305	0.0

Only the AMFZ samples were tested in this respect. The stability of the standard solution and the sample solution was studied for 4 days. Both solutions were stored in a refrigerator between 2-8 °C. The acceptance criterion was set up according to the relative difference value shown below:

$$\text{Relative difference} = \left| \frac{A_{Start} - A_{Stored}}{A_{Start}} \right| \cdot 100 \quad (6)$$

The solution was considered stable as long as the relative difference at a specific time point was not more than 3.0%. On the basis of the data presented in **Table 5,** the standard solutions can be considered stable for at least 96 h, and the sample solutions can be considered stable for at least 96 h.

Robustness

The effects of changing the organic–aqueous ratio, the pH of the aqueous phase, the flow rate of the mobile phase and the temperature of the column on the retention time and on the shape of the AMFZ and paracetamol peaks were examined. The results of the robustness studies presented in **Table S-3** demonstrate that the ratio of the aqueous and organic phases exerted a great influence on both the retention time and the peak symmetry of the analytes. Overlaid chromatograms of the robustness study in the case of AMFZ are shown in **Figure 10** to provide a more straightforward visual display of the tabulated data. It can be seen that the pH of the aqueous phase significantly changed the symmetry of the peak, which is in accordance with the results obtained from the simulations with the Pallas software. The lower the pH, the more asymmetrical the peak was. On the other hand, the pH of the mobile phase had only a very slight effect on the retention time of the peak. The flow rate influenced the retention time, as expected, while it had a negligible effect on the peak shape. The column temperature did not influence either the retention time or the symmetry of the main peak.

The overlaid chromatograms of the paracetamol robustness study are shown in **Figure 11**. Changing the pH of the aqueous component of the mobile phase did not influence the retention parameters of paracetamol significantly. On the other hand, higher temperature, a higher organic ratio in the eluent and a higher flow rate of the eluent significantly decreased the retention time. The change in the aqueous organic ratio affected the peak shape of paracetamol strongly, which changed from 0.911 to 1.215 during the experiments. In all other cases, the symmetry of the peak remained stable around 1.15.

It can be stated on the basis of the above data that the developed methods are robust within the studied parameter ranges.

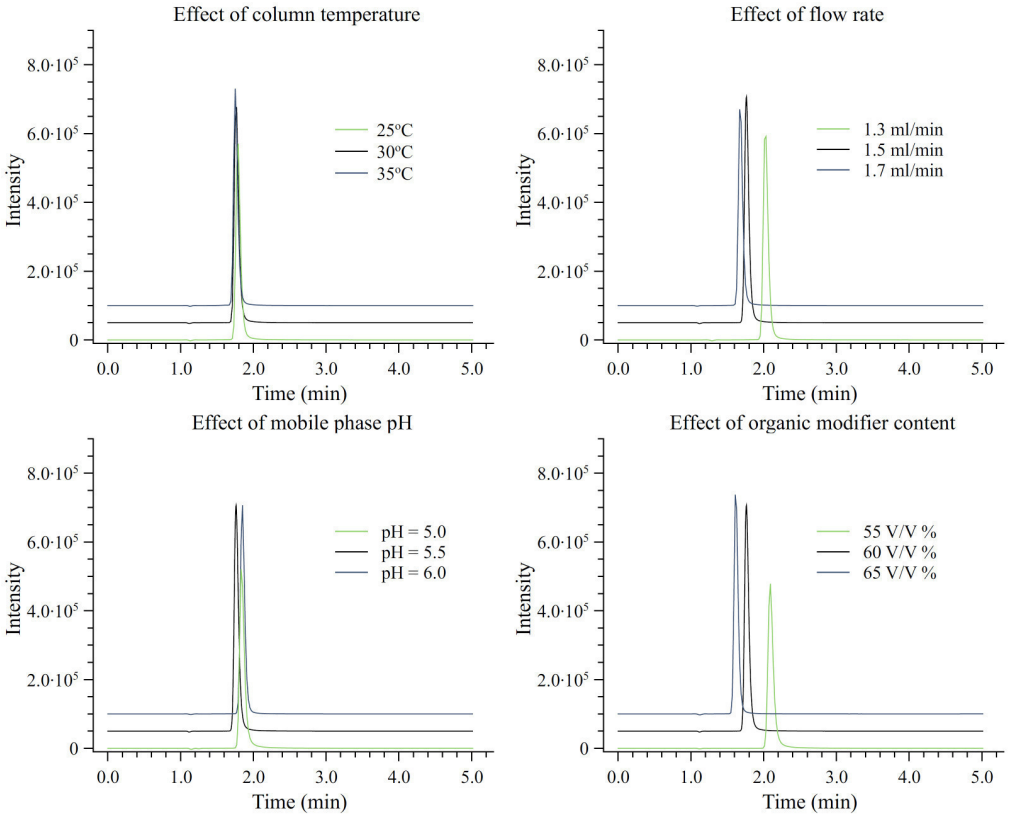


Figure 10 AMFZ robustness test results

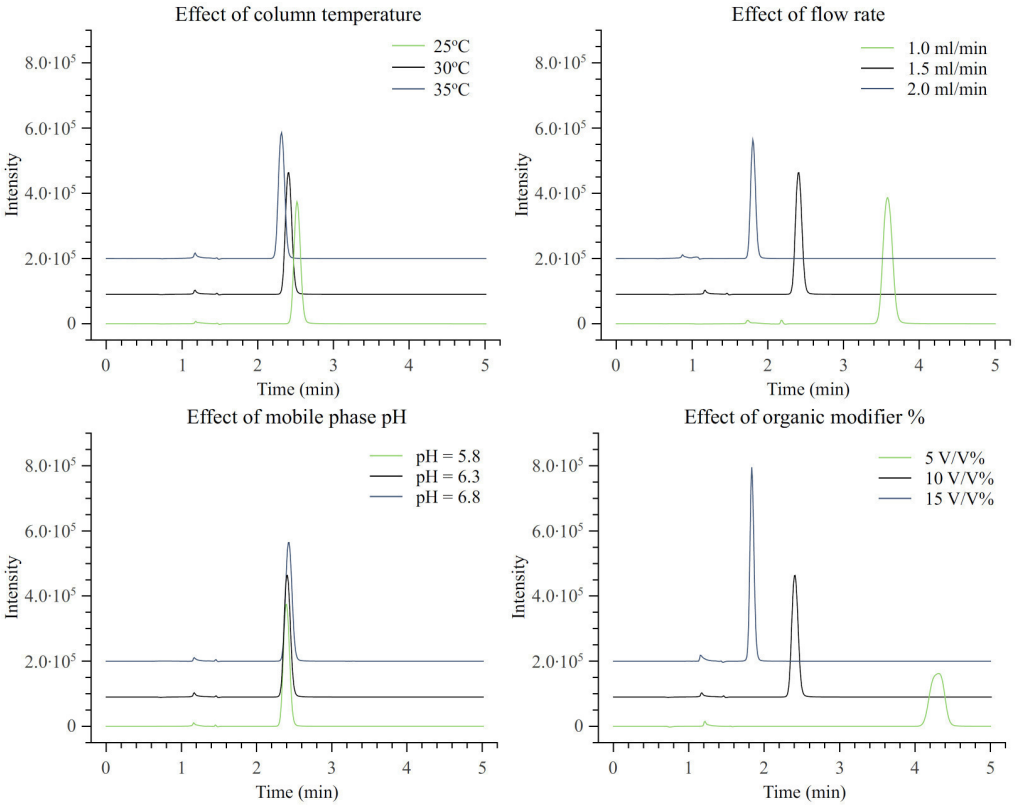


Figure 11 Paracetamol robustness test results

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

4.2.1. Suppositories without surfactants

Magistral prescriptions do not usually specify the suppository base to be used as vehicle and it is left to the pharmacists to apply their professional knowledge to choose the most suitable one from the possibilities listed in the official Pharmacopoeia. The development of the sample preparation involved in particular two suppository vehicles, adeps solidus and massa macrogoli, as these are the most commonly chosen ones. The same MeOH–water solvent mixture (50:50, V/V) was used for both vehicles. However, the methods differed as concerns other aspects of the sample preparation. This is due to the fundamentally different physico-chemical properties of these two vehicles.

Adeps solidus and massa macrogoli cannot be distinguished by purely organoleptic examination. In the first step of sample preparation, the suppository (containing the unindentified vehicle) was weighed in a beaker, 15 ml of the above solvent mixture was added, and the beaker was heated in a 40 °C water bath until the suppository melted. (At this point, the behaviour of the molten suppository revealed its nature. In the case of adeps solidus, a consistent, clear, colourless fatty phase appeared on the surface of the solvent mixture, whereas with massa macrogoli the solution became homogeneous and clear and no second phase could be observed. In some cases, massa macrogoli may contain a certain amount of tensides, when the resulting solution was opaque, but even then no second phase or fat droplets could be observed.) At this stage, the active substance was extracted from the vehicle by shaking the sample for 10 min.

The massa macrogoli-based samples did not require filtration, so the solution was transferred directly into a 50 ml volumetric flask and the beaker was rinsed with another 15 ml and then 2 x 5 ml of solvent mixture, the rinsing solvent likewise being transferred to the volumetric flask, the solution next being made up to volume with the solvent mixture.

The adeps solidus-based samples required removal of the fatty phase by freezing on an ice-bath, when the fat solidified and the liquid could be decanted into a 50 ml volumetric flask. This extraction step was repeated with a second 15 ml portion of solvent mixture in a 40 °C water bath. The beaker was finally washed twice with 5 ml of solvent mixture, which was transferred to the volumetric flask, the solution then being made up to volume with the

solvent mixture. The outstanding benefit of this sample preparation procedure is that it does not require an initial knowledge of the suppository base used.

Finally, in both cases a 0.3 ml aliquot of the stock solution was transferred to a 10 ml volumetric flask and made up to volume with the solvent mixture. The solution was filtered on a Millipore Millex PVDF membrane filter with a pore size of 0.45 μm.

4.2.2. Surfactant-containing suppositories

As W35TT is based on hard fat, it seemed reasonable to choose the sample preparation method described in section 4.2.1 for hard fat as the starting point for the development. However, our expectations were not fulfilled; the recovery of the API from the W35TT base was only 88%. It was hypothesized that the surfactants formed micelles within the suppository base, which encapsulated some of the API. The sample preparation procedure described in section 4.2.1 for hard fat was not suitable for quantitative release of the drug from the micelles for the analysis. This caused the difference in recovery between the two bases. In order to achieve a satisfactory release, the micelles had to be broken down to gain access to the entrapped drug. It was presumed that salting-out might be a suitable method to solve this problem.

4.2.2.1. Effect of sodium chloride concentration on drug recovery

The procedure applied in the case of hard fat was amended with an additional step by adding sodium chloride solution in increasing concentrations to the first 15 ml of solvent in order to study the micelle-opening effect of salting-out. The sodium chloride concentrations applied were 0, 50, 100, 150 and 200 mM. At all levels, two parallel samples were prepared. HPLC analysis revealed that the recovery from all of the samples remained below 95%. **Figure 12** demonstrates that the increasing sodium chloride concentration did not correlate with the observed recovery change. Calculated covariance between the concentrations and the recoveries suggested a slightly increasing relationship, which was possibly due to the minor CMC-decreasing effect of strong electrolytes [103, 104]. The question may arise as to whether further increase of the sodium chloride concentration might have improved the recovery sufficiently. The answer is a clear no because higher sodium chloride concentration would probably have caused the precipitation of the salt in the mobile phase as sodium chloride has a solubility one order of magnitude lower in MeOH than in water, making the

analysis impossible. Precipitation could have damaged the silica core of the column, or could have caused clogging in the flow lines; therefore, higher salt concentrations were not tested.

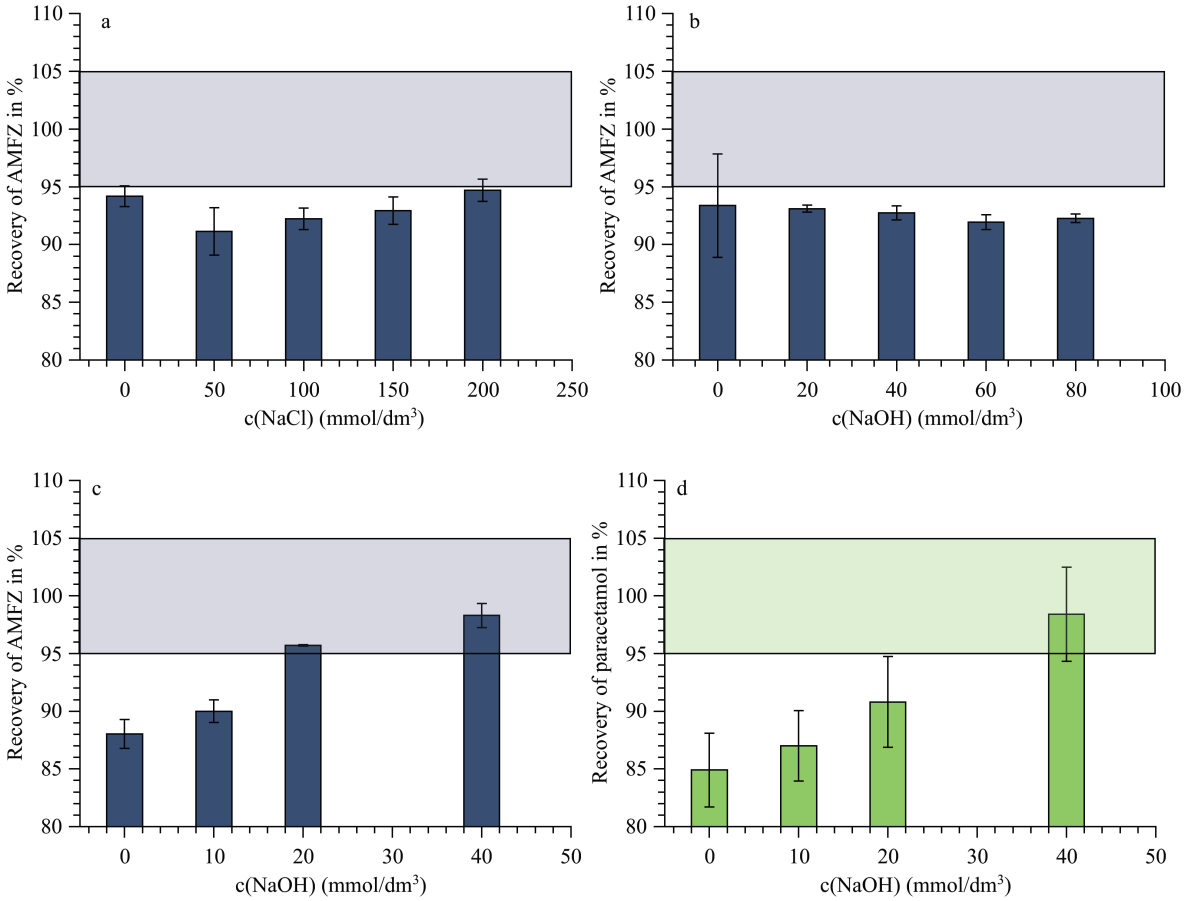


Figure 12 Recovery of AMFZ and paracetamol. (a) Effects of NaCl concentration. (b) Effects of pH. (c,d) Effects of pH at constant c(NaCl) = 100 mM. Vertical bars denote means of 3 independent measurements (n=3), error bars indicate the standard deviation of the 3 data. Covariances between the independent variable (concentration) and the dependent variable (recovery) for plot a=28.67; b=-13.47; c=58.71 and d=75.38

4.2.2.2. Effect of pH change on drug recovery

Micelle formation can be influenced by change of pH [105]. The API recovery was therefore studied in analytical samples containing increasing sodium hydroxide concentration. The additional step in this case was the addition of 5 M sodium hydroxide solution to the initial 15 ml of extraction solvent during the melting of the suppository. Three parallel samples were prepared, in which the concentrations were 0, 20, 40, 60 and 80 mM. The HPLC analysis indicated that none of the recovery results reached the lower limit of the required range of 95-105% at any sodium hydroxide concentration. Similarly to the sodium chloride concentration study, these recovery values did not exhibit any correlation with the

increasing base concentration. The covariance of the series of data (see caption of **Figure 12**) indicates a negative relationship. Therefore, higher sodium hydroxide concentrations were not studied.

4.2.2.3. Effect of US treatment on drug recovery

Micelles can be effectively destroyed by US treatment. Due to the ineffectiveness of pH change and salting out, 30 min ultrasound was applied on 3 parallel preparations of suppositories. The average recovery was 92.6±2.8%. Due to the fact that during longer US treatment the transfer medium underwent warming, longer agitation times were not applied. The ineffectiveness of the individual effects described in sections 4.2.2.1, 4.2.2.2 and 4.2.2.3 led to the straightforward idea to study the combined effects of sodium chloride, sodium hydroxide and US treatment simultaneously.

4.2.2.4. Combined effect of sodium chloride and sodium hydroxide on drug recovery

The combination of sodium chloride and sodium hydroxide was tested together with an additional 30 min US treatment. Constant, 100 mM sodium chloride and 0, 10, 20 or 40 mM sodium hydroxide were applied. The recovery results were favourable at 20 and 40 mM sodium hydroxide concentrations: 95.7% and 98.0%, respectively. **Figure 12** clearly reveals the correlation between the sodium hydroxide concentration and the recovery of the API when sodium chloride was present in the sample. The application of 100 mM sodium chloride and 40 mM sodium hydroxide was suggested.

This sample preparation method was also tested on paracetamol. Samples were prepared by the combined sodium chloride and sodium hydroxide addition method described in the previous paragraph. The suppository was modelled by combining the blank hard fat suppository, Tween 20 and Tween 60 in the appropriate proportion and exactly weighed 100.0 mg of paracetamol. The sodium hydroxide concentration was set to 0, 10, 20 or 40 mM during the experiment. The recoveries determined by HPLC analysis are presented in **Figure 12,** plot d. In the case of paracetamol, 40 mM sodium hydroxide was required to liberate the API entirely. The average recovery was found to be 98.4%.

The covariance values for plots c and d in **Figure 12** reveal a strong positive relationship between the increase of the hydroxide concentration and the change in the recovery. These values are significantly higher than those determined for plots a and b in **Figure 12**.

4.2.2.5. Extension of sample preparation method developed in section 4.2.1

On the basis of the results described in subsection 4.2.2.4, the sample preparation method shown in section 4.2.1 needed to be amended with the following extra step.

2 ml of 1 M NaOH and 1 ml of 5 M NaCl solution were added to the initial 15 ml of solvent mixture and the solution was sonicated for 30 min. After this, the sample preparation procedure remained similar to that described in section 4.2.1. The scheme of micelle breaking can be seen in **Figure 13**.

In order to find evidence of micelle formation in the sample solution of W35TT, the determination of CMC for both surfactants became necessary. The results of these studies are presented in the following section.

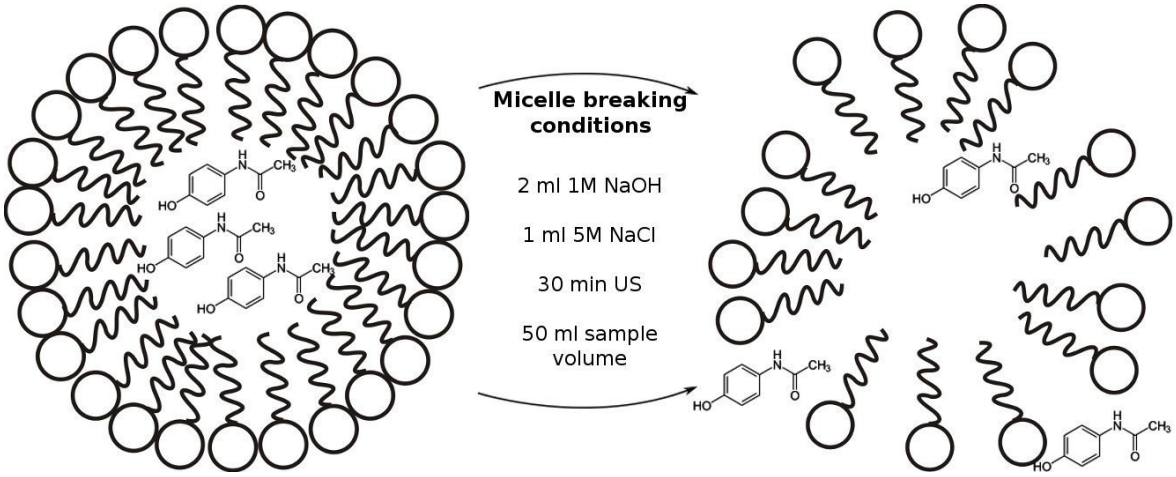


Figure 13 Theoretical figure of micelle-breaking mechanism

4.2.3. CMC determination: CMCs of TWEEN 20 and TWEEN 60

The concentration range for the combined surfactants was determined from the data for the initially characterized individual Tween 60 and Tween 20. The highest concentration in the series was always 0.5 mM, which corresponds to that of both surfactants in the sample solution in the analytical method for suppositories containing W35TT.

The absorbances of the series of solutions were determined at 550 nm at ambient temperature. This wavelength was chosen on the basis of preliminary experiments. A solution was prepared in which the surfactant concentration corresponded to the composition of the sample solution and a spectrum was acquired between 200 and 600 nm. In the spectrum, the wavelength for the study was chosen from a plateau region where the absorption was

independent of the wavelength. This indicated that the increase in absorbance is due to scattering rather than absorption. The absorbances obtained in this way were plotted against surfactant concentrations. The linear regions of the plots were fitted by straight lines and the intersection of the straight lines gave the concentration of the CMC. The CMC can be identified as the breakpoint of the absorbance vs. concentration curve [69, 106].

Table 6 Surfactant concentration ranges of CMC determination

	Concentration/mM															
Tween 60	0	0.03	0.04	0.05	0.06	0.08	0.09	0.1	0.2	0.3	0.5					
Tween 20	0	0.04	0.05	0.06	0.07	0.08	0.1	0.3	0.5							
Tween 20 & 60	0	0.001	0.005	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1	0.3	0.5			
Tween 20 & 60 with NaOH	0	0.001	0.005	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1	0.3	0.5	0.7	0.8	0.9

The CMCs of Tween 20 and Tween 60 were determined in the applied solvent mixture: MeOH : water (50:50 V/V) in the presence of hard fat by measuring the turbidity of solutions with different surfactant concentrations at 550 nm. Four series of solutions were prepared with a constant amount of hard fat and increasing concentrations of the two surfactants, either separately or together, and together in the presence of NaOH. The concentrations used are listed in **Table 6**.

Table 7 Calculation of CMCs from the data of fitted straight lines

	Slope		SD	Intercept		SD	Intersect/mM
Tween 20	0.4	±	0.110	0.24	±	0.033	0.084
	2.8	±	0.485	0.04	±	0.027	
Tween 60	0.52	±	0.039	0.12	±	0.011	0.061
	4.1	±	1.022	-0.096	±	0.047	
Tween 20 & Tween 60	2.1	±	0.163	0.75	±	0.040	0.040
	12.6	±	2.083	0.34	±	0.032	
Tweens 20 & 60 with salt and base	0.07	±	0.011	1.99	±	0.199	0.324
	0.529	±	0.008	-0.09	±	0.037	

Turbidity was plotted against surfactant concentration. Straight lines were fitted to each series of points. The parameters of the fitted straight lines and their intersects are presented in

Table 7. In MeOH : water (50:50, V/V) as solven,t the CMCs of Tween 20, Tween 60 and their mixture were 0.084 mM, 0.061 mM, and 0.040 mM, respectively.

These results are in good agreement with the trends reported in previous studies. The slight differences from the literature data (**Table 1**) can be attributed to the different measurement techniques and the different solvents [59, 62]. The solvent in this case, an equivolume mixture of water and MeOH, could increase the CMC. Short-chain alcohols such as MeOH or ethanol are known to decrease the hydration of the ethylene oxide chains of non-ionic surfactants, so the observed trend in the CMC increase is in good agreement with this explanation [107]. The tendencies earlier found for different Tween substances can be observed in our results too: the CMC of Tween 20 is higher than that of Tween 60. Moreover, the two surfactants together give a significantly lower CMC, which is also characteristic [58]. It is obvious that the applied surfactant concentration in the suppository base is well above the determined CMC, which explains the micelle formation and the decreased recovery of drug as compared with the "pure" hard fat. Although the application of surfactants in the suppository base has a beneficial effect on the formulation properties of the material and on the drug release in the body, the amounts of surfactants must be optimized to avoid the analytical pitfall of failing the recovery study. Too high a surfactant concentration will always pose a potential risk of analytical error.

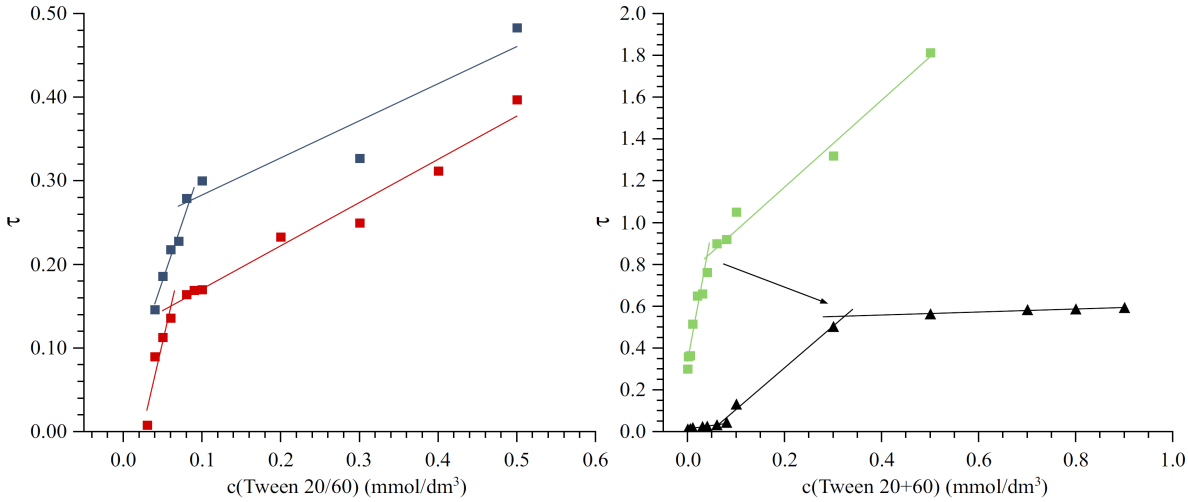


Figure 14 Turbidimetric plots for determination of CMCs of Tween 20 (▪), Tween 60 (▪), Tween 20 & 60 (▪) and Tweens 20 & 60 with salt and base (▪)

The complex sample preparation method, which was tested with two different model compounds, is rapid and effective in the case of suppository bases containing surfactants to enhance the liberation of the encapsulated drugs. The success of the development can be

explained through the micelles losing their colloidal stability due to the loss of electrostatic interactions, which is caused by the addition of electrolytes. The mechanism is similar to that in formulations with targeted drug release. The initial hypothesis was proven by determining the CMC of Tween 20 and Tween 60 in a medium where the final sodium chloride concentration was 100 mM and the sodium hydroxide concentration was 40 mM. The results can be seen in **Figure 14**. The CMC of the combined surfactants is about 5 times higher in the presence of salt and base than in their absence. The applied salt and base concentrations combined with the US treatment was sufficient to raise the CMC of the system so that the surfactants within the suppository could not assemble into micelles entrapping part of the API.

4.2.4. Stability verification of the drugs by NMR spectroscopy during sample preparation

The stability of the prepared samples was verified to make sure that the applied sample preparation method did not cause any degradation or decomposition of the active substance (AMFZ or paracetamol) and that its structure remained unchanged throughout the experiment. The measurements were carried out at 25 °C.

The analytical sequence for both model compounds incorporated the measurement of a reference solution, which contained the appropriate compound in the solvent used during the chromatographic sample preparation, and the sample solution itself. The method of sample preparation for the HPLC measurements had to be altered slightly as the NMR studies required higher concentrations of the active substances. A 5-fold amount of AMFZ or paracetamol (500 mg) was therefore dissolved in the appropriate solvent described in section 4.2.1 to 50 ml in the case of the reference solutions. The sample solution was prepared similarly to the reference solution, except that 1 W35TT blank suppository was dissolved in the solvent together with 1 ml of 1 M sodium hydroxide and 1 ml of 5 M sodium chloride solution before making the solution up to volume. For the paracetamol measurements, 500 mg of substance was dissolved for the reference solution in 50 ml of solvent described in section 4.2.1. The solution modelling the sample was prepared by dissolving 500 mg of paracetamol, and placing 1 blank W35TT suppository in the first 15 ml of solvent. Micelle demulsion was achieved according to subsection 4.2.2.5, except for the last dilution step. 10 V/V% of D_2O was added to each sample before the NMR measurements.

In the assigned spectra of paracetamol, the signals of the OH and NH protons are not visible due to the fast exchange with water. It is clearly visible in the paracetamol sample

spectrum that, due to the deprotonation of the OH and NH groups with the increase of pH, the chemical shift of the aromatic protons decreases by 0.2 ppm. In the case of AMFZ, no deprotonation takes place, and thus there is no change in the chemical shifts.

The overlaid spectra showed no significant difference, i.e. no new peaks were detected, which confirmed that the samples remained intact during the sample preparation (**Figure 15**).

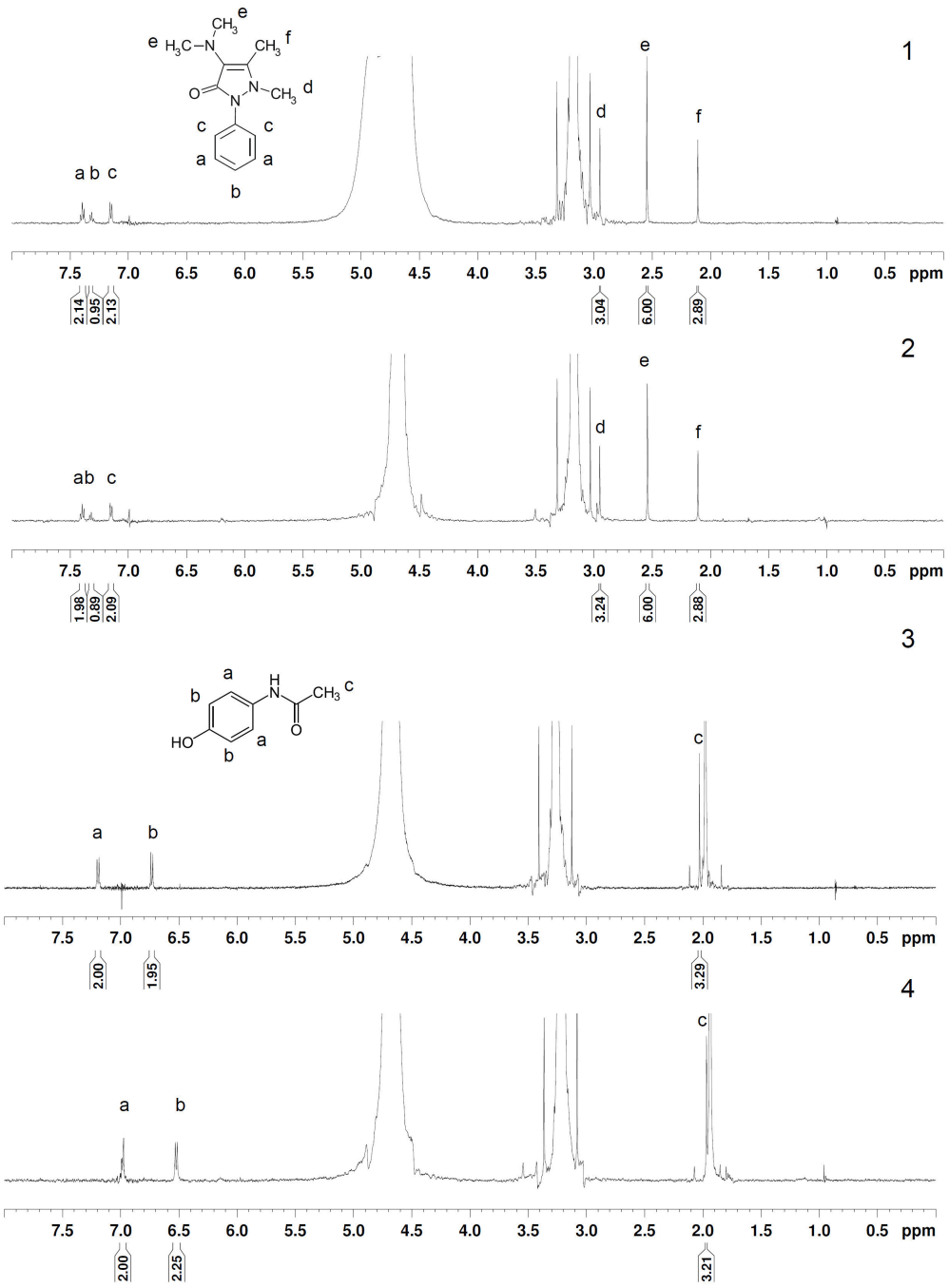


Figure 15 ^{1}H NMR spectra of aminophenazone and paracetamol standards and samples. The signals marked with letters prove that no decomposition takes place in the sample solution treated with strong base. Peaks a and b of paracetamol are shifted to the right by 0.2 ppm due to the deprotonation of the OH and NH groups in the alkaline medium

4.2.5. Dissolution tests of hard fat and W35TT suppositories

Dissolution tests were carried out in a Hanson SR8+ dissolution tester equipped with a basket apparatus. Commercially available suppositories (prepared for children) containing aminophenazone and either hard fat or W35TT were tested. The dissolution medium, potassium dihydrogenphosphate buffer (pH 7.5; 0.05 M), was thermostated to 37 °C. The agitation speed was 50 rpm. Samples were collected at 5, 10, 15, 30, 45, 60 and 120 min and tested by HPLC with the system described in section 2.2.3.

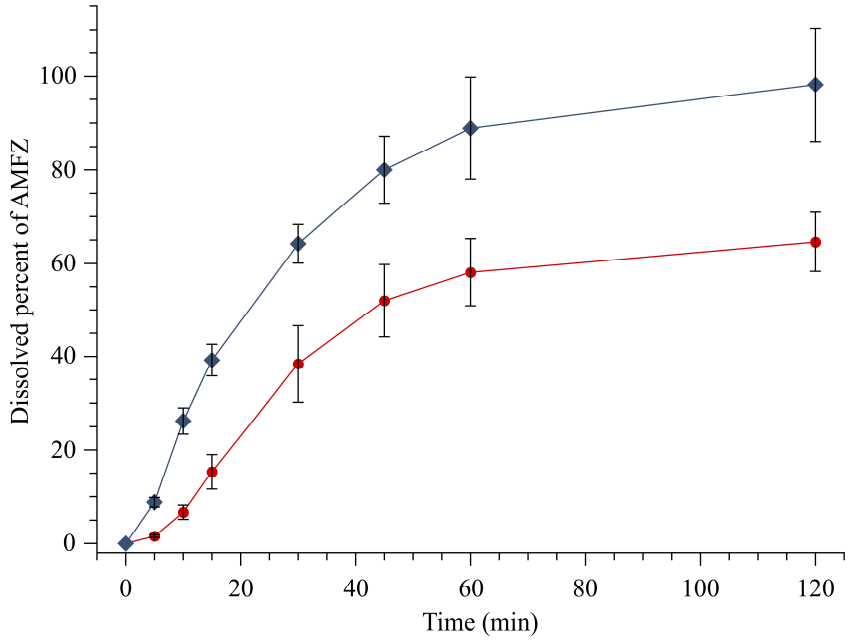


Figure 16 Dissolution profiles of AMFZ containing hard fat (●) and W35TT (♦) suppositories

Six dosage units were tested from both hard fat and W35TT-containing suppositories according to the parameters described in section 2.2.7. It can be seen in **Figure 16** that dissolution from solid fat-based suppositories is slower in the time range critical for the *in vivo* effect, and up to 120 min only 70% of the drug is released. W35TT-based suppositories demonstrated saturation from 60 min (88.9±10.9%). On the other hand, it should be noted that during these experiments the volume of the dissolution medium was 900 ml, where the concentration of Tween 20 was 0.0244 mM and that of Tween 60 was 0.0228 mM, which was significantly lower than in the case of our analytical samples. Application of such large volumes for sample preparation to avoid micelle formation is not recommended in routine laboratories. Furthermore, it is worth consideration that the dissolution rate in large volumes is favourably influenced by surfactants because their concentration falls below their CMC, but this certainly cannot model the physiological conditions, where the volume of rectal fluid is

only 2-3 ml. In such a small volume, the surfactant concentration will be definitely higher than those in our analytical samples and will fall above the CMC in every case. If a 2.5 ml rectal fluid volume is hypothesized, the concentration of Tween 20 will be 9.76 mM and that of Tween 60 will be 9.12 mM, accounting for an even higher extent of micelle formation.

4.2.6. Extension of the validation study with matrix-dependent performance characteristics

Precision/ Repeatability

Repeatability was checked on 6 individual AMFZ or paracetamol suppositories prepared according to the developed methods described in sections 4.2.1 and 4.2.2.5. In the case of adeps solidus as vehicle, 1 of the 6 replicate results exceeded the 125% limit, and this result was omitted from the calculation of the RSD% on the basis of our result that there is no carryover between the injections and the fact that active substance was not added to the solution. RSD% proved to be 1.4%, which can be considered acceptable when it is taken into account that not composite samples of multiple dosage units, but rather unique dosage units were used for each sample preparation. The massa macrogoli-based suppositories gave an RSD% of 2.1% and the AMFZ W35TT samples had an RSD% of 1.5%, which are also acceptable. The other model system (paracetamol W35TT) results are similarlye acceptable, because the RSD% is 4.4%.

Precision/ Intermediate precision

The same analysis procedure was carried out by another analyst on another day, using a freshly prepared mobile phase.

$$\text{Relative difference} = \frac{\left|\overline{X}_{\text{Dayl}} - \overline{X}_{\text{Day2}}\right|}{\overline{X}_{\text{Dayl}} + \overline{X}_{\text{Day2}}} \cdot 2 \cdot 100\% \qquad (5)$$

The results for the adeps solidus-based samples of AMFZ were an RSD% of 1.2% and a relative difference of 1.3% between the averages of the repeatability (Day 1) and intermediate precision (Day 2) results compared to the mean of the average values measured for each. Both results can be accepted according to the principles of general pharmaceutical analytical practice. For the massa macrogoli-based samples of AMFZ, the RSD% of the individual results was 2.5%, while the relative difference between the repeatability and intermediate precision was 3.7%. The samples of the third vehicle, W35TT with AMFZ, gave an RSD% of

1.7%, while the relative difference was found to be 5.5%. These values for W35TT samples of paracetamol were an RSD% of 4.9% and a relative difference of 2.9%. All four results were in accordance with the appropriate guidelines, and were therefore considered acceptable.

Accuracy

The accuracy of the AMFZ method was studied between 50% and 450% of the nominal content of the suppositories, i.e. 100 mg, except for W35TT, which was studied between 50% and 300%. The results are shown in **Table S-4**. Although all of the average values fell between 95% and 105%, it should be mentioned that in the cases of adeps solidus and W35TT most of the averages were below 100%, while in the case of massa macrogoli they were above 100%. This may raise a warning flag, but there was no trend within the results that could be correlated with the increasing concentration of the sample groups.

The accuracy of the paracetamol method with respect to the W35TT base was studied at 50%, 100%, 150% and 200% concentration levels with three parallel samples. The results fell in the range 100.9-103.2%, which is acceptable. The results are shown in **Table S-5**.

Specificity

When the procedure was carried out with blank suppositories (containing no active substance), no peak was detected at the retention times of AMFZ or paracetamol. It can be stated that there are no excipients in either vehicle that interfere with the determination of AMFZ or paracetamol.

4.3. Part III. Quantitative analysis of magistrally produced suppositories

In this section, an investigation of samples prepared in pharmacies is reported, with special emphasis on the homogeneity and the total API content of the batches. The circumstances of the preparation of the suppositories were known in all cases and are presented.

4.3.1. Comparison of the assay results obtained with cerimetric titration and HPLC

Two analytical methods were first compared by measuring 6 samples each from the same factory-produced batch, and the individual assays of the suppositories were carried out by either the volumetric titration (see section 3.2.2) or the chromatographic technique.

Samples of extemporaneously prepared suppositories for HPLC analysis were prepared according to the flow chart in **Figure 17**.

All of the final results (the averages of the 3 replicates in the case of the titrimetric method, and the assay value in the case of the chromatographic determination) fell within the range 95-105%, which conforms to the strictest requirements of the EP. The individual content data are presented in **Table 8.**

Table 8 Assay results on factory-produced suppository samples, measured by titrimetry or HPLC

Sample	Cerimetric titration				HPLC assay
	Replicates %			Content %	Content %
1	101.6	107.2	103.5	104.1	103.9
2	98.9	99.3	97.5	98.5	104.9
3	106.3	100.7	100.3	102.4	102.8
4	96.1	100.7	97.1	98.0	102.3
5	100.9	110.4	102.1	104.5	103.9
6	103.6	104.0	104.7	104.1	104.6
Mean	-	-	-	**101.9**	**103.7**
SD	-	-	-	**2.93**	**1.01**

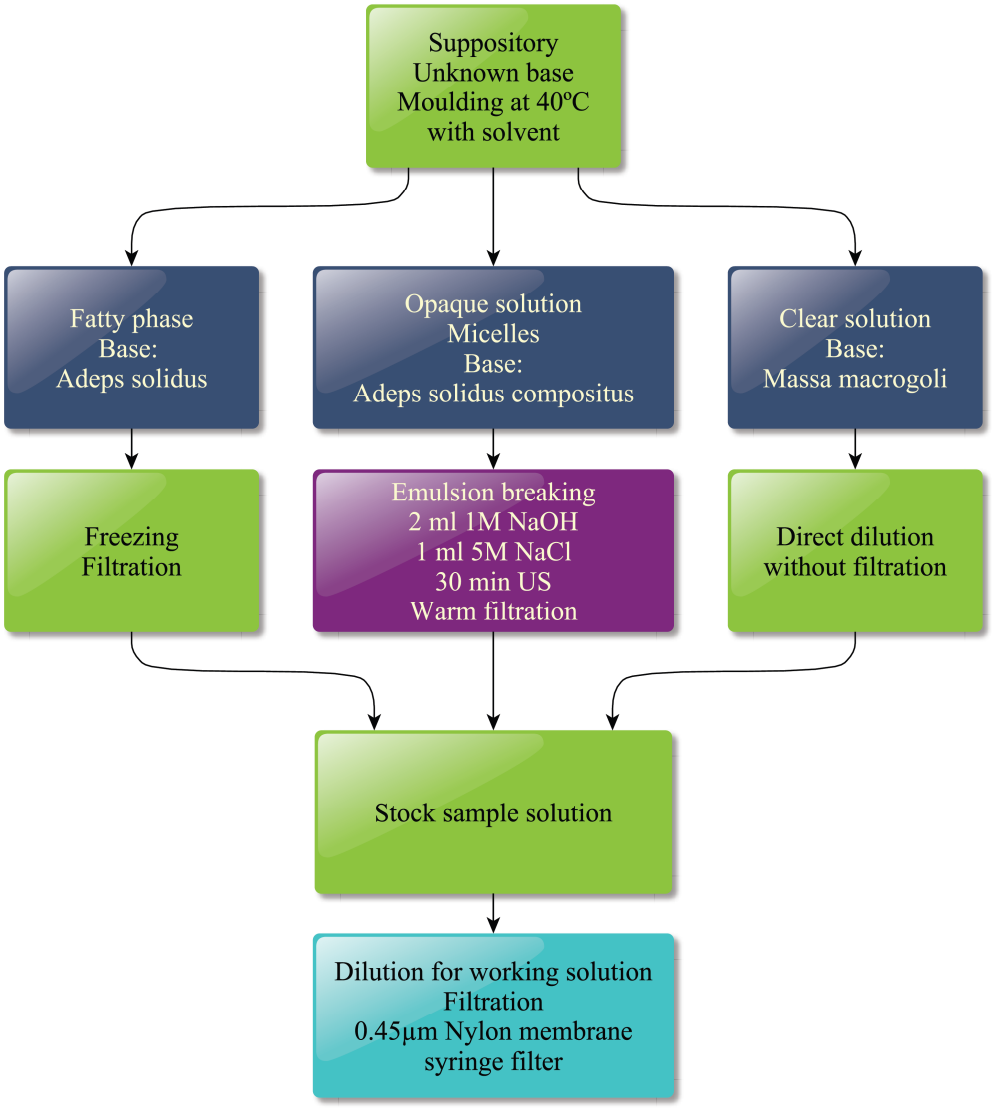


Figure 17 The flow chart of the sample preparation procedure

It should be noted that the volumetric results exhibit a larger standard deviation than that of the chromatographic results, but the preparation of the sample for titrimetry (replicate samples are prepared from the molten suppository) involves a higher level of uncertainty, and this can therefore be considered acceptable. Comparison of the two methods reveals that both can be used for the analysis of suppositories containing AMFZ.

4.3.2. Dosage uniformity study of magistrally produced suppositories

We additionally studied paediatric suppositories with the composition described in section 3.1. All samples were from different Hungarian public pharmacies, and each sample consisted of 10 individual suppositories. The manufacturing technology of each of the

samples was known to us; the pharmacists committed intentional technological errors during the preparation of the suppositories. Samples Ph1-Ph9 were measured with the HPLC technique described in subsection 4.1.2.2, while samples Ph10-Ph15 were tested for dosage uniformity with the titrimetric method presented in section 3.2.2. Ph in the sample identifier stands for pharmacy. All of the findings were compared (see **Figure 18** and **Table 9**) on the basis of the results given in section 4.3.1. It can be stated that 2 samples conformed to the specifications of EP 7.8, i.e. the individual assay values fell within the range 85-115% at level 1, and 1 further sample would probably conform to the level 2 specification of 75-125%. For the remaining samples, generally either lower individual assay results or (in 2 samples) significant inhomogeneity was found.

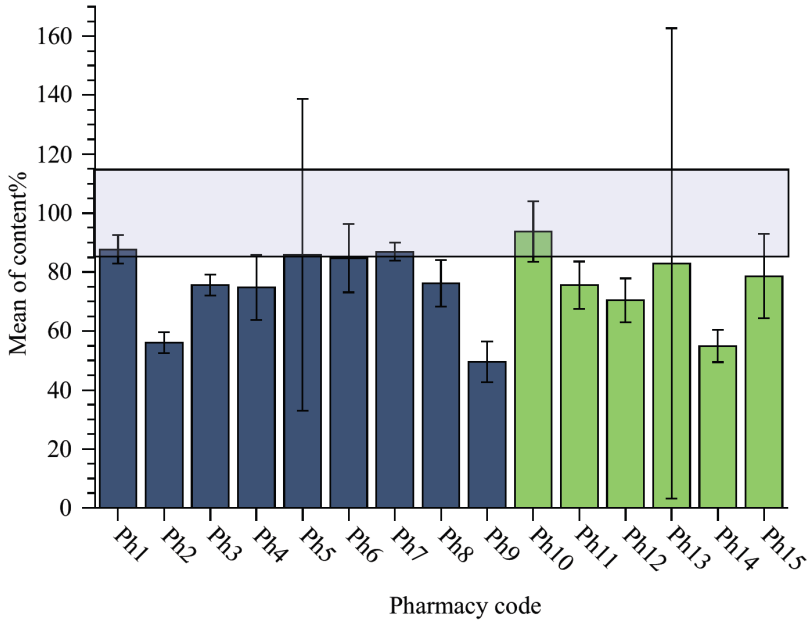


Figure 18 Mean API contents for the samples, with the standard deviations. An API content in the interval 85-115% is satisfactory. Samples Ph1-Ph9: measured by HPLC; samples Ph10-Ph15 measured by cerimetric titration

4.3.3. Effects of *f* on the assay results

The possible consequences of the most common errors can be illustrated on a theoretical example. If the pharmacist produces suppositories on the basis of the following parameters: E = 1.7 g f = 0.78 and s = 0.1 g, then, according to Equation 1, T_m = 15.22 g for 10 suppositories. If f is not applied, but only the weight of the API is subtracted from the value of E, then we heve T_m = 16.0, which will result in an assay 4.6% lower than the required value. If this error is superimposed with the one when the calculated moulding excess is taken solely

from the suppository base, by taking the required base for 12 dosage units instead of 10, the concentration of 1 suppository is diluted even further, to 80.3% of the intended theoretical value. Such errors are visible in case of samples Ph3, Ph8, Ph11 or Ph12.

4.3.4. Effects of stirring on the homogeneity and total assay of the samples

The suppository mass can be homogenized well by choosing an appropriate rate of manual stirring or machine-based mixing. Stirring during the moulding process can help avoid sedimentation of the active substance in the container. Too slow stirring is not effective, but too fast stirring may also lead to errors: air bubbles are formed in the mass, which will decrease the weight of the suppositories, foam can be formed from surfactant-containing bases, or shearing forces may appear, which decrease the viscosity of the suppository mass by rheodestruction, causing the rapid sedimentation of the active substance. The ideal machine stirring speed for fat-based suppositories is 150 rpm.

Table 9 Average assay results on the samples and standard deviations in the homogeneity study

Sample	Ph1	Ph2	Ph3	Ph4	Ph5	Ph6	Ph7	Ph8	Ph9	Ph10	Ph11	Ph12	Ph13	Ph14	Ph15
Average %	87.7	56.1	75.6	74.8	85.8	84.7	86.9	76.2	49.5	93.7	75.5	70.4	82.9	54.9	78.6
SD	4.8	3.6	3.6	11.1	52.8	11.6	3.0	7.8	7.0	10.2	8.0	7.4	79.7	5.5	14.3

The results demonstrate that the stirring technique of the preparing pharmacist before and during the moulding procedure was appropriate, with the exception of a small number of serious cases. Those samples can be considered homogeneous which gave SD $\leq$ 10 with respect to the individual suppository assays. A larger deviation can originate from the lack of stirring during the moulding, as may be seen for samples Ph4, Ph6 and Ph15. Extremely large deviations result when both thorough homogenization and stirring during the moulding process are omitted, which may be observed for samples Ph5 and Ph13. If *f* is not applied and the suppository base is applied in excess amount, the assays of the samples will fall below the lower limit of acceptance. If the molten mass is not stirred during moulding, the decrease in the active substance content becomes more serious as the suspended material is sedimented, and the mould will contain an active substance-depleted mixture. Such samples were Ph2, Ph9 and Ph14.

5. Final conclusions

5.1. 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.

5.2. 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.

5.3. 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.

Summary

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, 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.
   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 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.
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.

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 conditions described in section 3.3.

3.5. In a comparative dissolution study of adeps solidus and W35TT-based suppositories, dissolution samples were analysed with the HPLC method developed in section 2.1. 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.

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.

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 total 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.

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SUPPLEMENT

Table S-1 Results of accuracy studies

Level		Paracetamol %			ASA %			Papaverine %		
		Rep.	Mean	RSD	Rep.	Mean	RSD	Rep	Mean	RSD
70%	1.	103.0	102.5	0.45	100.8	100.1	0.61	98.4	98.1	0.26
	2.	102.1			99.8			97.9		
	3.	102.3			99.7			98.2		
100%	1.	101.4	101.9	0.43	98.3	98.5	0.17	98.2	98.7	0.47
	2.	102.3			98.7			98.9		
	3.	102.1			98.5			99.0		
130%	1.	100.7	101.7	0.94	97.0	97.6	0.59	95.8	96.8	0.90
	2.	102.5			98.1			97.4		
	3.	102.1			97.7			97.1		

Table S-2 Results of method robustness tests

Condition changed		Paracetamol			ASA			Papaverine		
		t_R/min	N	Symmetry factor	t_R/min	N	Symmetry factor	t_R/min	N	Symmetry factor
Column temperature/°C	55	2.660	6380	1.261	6.324	69377	1.445	7.122	50747	1.597
	60	*2.527*	*5540*	*1.237*	*6.213*	*69445*	*1.460*	*7.160*	*53197*	*1.588*
	65	2.358	5600	1.264	6.032	81057	1.423	6.992	60963	1.542
Buffer pH	3.23	2.531	5843	1.249	6.308	68186	1.476	6.933	53433	1.629
	3.43	*2.527*	*5540*	*1.237*	*6.213*	*69445*	*1.460*	*7.160*	*53197*	*1.588*
	3.63	2.516	5690	1.245	6.072	75013	1.412	7.252	52516	1.563
Flow rate/(ml/min)	1.4	2.695	5521	1.242	6.453	68816	1.437	7.459	51720	1.604
	1.5	*2.527*	*5540*	*1.237*	*6.213*	*69445*	*1.460*	*7.160*	*53197*	*1.588*
	1.6	2.357	5346	1.265	6.000	70952	1.449	6.900	53720	1.595
Aqueous : organic ratio	5%	3.030	6408	1.210	6.324	77103	1.418	7.132	54830	1.583
	7%	*2.527*	*5540*	*1.237*	*6.213*	*69445*	*1.460*	*7.160*	*53197*	*1.588*
	9%	2.122	5865	1.319	5.986	69115	1.476	6.947	54415	1.567
	23%	2.516	6052	1.270	6.399	65585	1.404	7.714	45410	1.613
	25%	*2.527*	*5540*	*1.237*	*6.213*	*69445*	*1.460*	*7.160*	*53197*	*1.588*
	27%	2.502	6152	1.252	5.998	78520	1.443	6.618	63169	1.559

Table S-3 Results of robustness studies. The second line of every condition changed refers to the nominal value of the parameter

Condition changed	AMFZ method				Paracetamol method			
		t_R/min	N	Symmetry factor		t_R/min	N	Symmetry factor
Aqueous : organic ratio	**45:55**	2.088	3973	1.535	**95:5**	4.309	2512	0.911
	40:60	1.761	4074	1.434	**90:10**	2.400	2887	1.148
	35:65	1.616	4512	1.460	**85:15**	1.803	3746	1.215
Buffer pH	**5.00±0.05**	1.837	3747	1.602	**5.80±0.05**	2.391	2948	1.146
	5.50±0.05	1.761	4074	1.434	**6.30±0.05**	2.400	2887	1.148
	6.00±0.05	1.846	4441	1.346	**6.80±0.05**	2.421	2906	1.150
Flow rate (ml/min)	**1.3**	2.021	4346	1.432	**1.0**	3.575	3458	1.132
	1.5	1.761	4047	1.434	**1.5**	2.400	2887	1.148
	1.7	1.676	4117	1.405	**2.0**	1.799	2495	1.175
Column temperature/°C	**25**	1.785	3890	1.433	**25**	2.514	3134	1.168
	30	1.761	4074	1.434	**30**	2.400	2887	1.148
	35	1.751	4340	1.408	**35**	2.308	2873	1.119

Table S-4 Results of AMFZ accuracy studies

Level	Adeps solidus				Massa macrogoli			W35TT		
	Replicates		Mean	RSD%	Replicates	Mean	RSD%	Replicates	Mean	RSD%
50%	1.	98.7%	99.4%	0.63%	104.10%	102.5%	1.56%	102.3%	98.8%	3.24%
	2.	99.9%			100.90%			96.0%		
	3.	99.6%			102.40%			98.2%		
100%	1.	99.5%	100.3%	0.75%	97.40%	99.7%	1.99%	97.7%	99.5%	2.71%
	2.	100.3%			100.50%			98.2%		
	3.	101.0%			101.10%			102.6%		
150%	1.	96.0%	95.3%	1.36%	104.60%	104.9%	0.42%	101.3%	99.7%	2.05%
	2.	93.8%			105.40%			100.4%		
	3.	96.1%			104.70%			97.4%		
300%	1.	96.2%	95.5%	0.87%	104.60%	102.7%	2.43%	100.1%	99.2%	1.84%
	2.	95.8%			103.70%			97.1%		
	3.	94.6%			99.90%			100.4%		
450%	1.	96.3%	96.5%	0.55%	102.80%	102.5%	1.49%	-	-	-
	2.	96.1%			103.80%			-		
	3.	97.1%			100.80%			-		

Table S-5 Results of accuracy measurement of paracetamol in W35TT

Level	Replicates recovery (%)		Mean (%)	RSD%
50%	1.	95.4	101.1	6.8
	2.	108.7		
	3.	99.1		
100%	1.	96.9	99.2	3.3
	2.	97.8		
	3.	103.0		
150%	1.	102.1	103.2	1.1
	2.	103.2		
	3.	104.3		
200%	1.	105.0	100.9	4.9
	2.	102.2		
	3.	95.4		

APPENDIX

I.

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Novel sample preparation method for surfactant containing suppositories: Effect of micelle formation on drug recovery

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ABSTRACT

Rectal drug delivery is currently at the focus of attention. Surfactants promote drug release from the suppository bases and enhance the formulation properties. The aim of our work was to develop a sample preparation method for HPLC analysis for a suppository base containing 95% hard fat, 2.5% Tween 20 and 2.5% Tween 60. A conventional sample preparation method did not provide successful results as the recovery of the drug failed to fulfil the validation criterion 95–105%. This was caused by the non-ionic surfactants in the suppository base incorporating some of the drug, preventing its release. As guidance for the formulation from an analytical aspect, we suggest a well defined surfactant content based on the turbidimetric determination of the CMC (critical micelle formation concentration) in the applied methanol–water solvent. Our CMC data correlate well with the results of previous studies. As regards the sample preparation procedure, a study was performed of the effects of ionic strength and pH on the drug recovery with the avoidance of degradation of the drug during the procedure. Aminophenazone and paracetamol were used as model drugs. The optimum conditions for drug release from the molten suppository base were found to be 100 mM NaCl, 20–40 mM NaOH and a 30 min ultrasonic treatment of the final sample solution. As these conditions could cause the degradation of the drugs in the solution, this was followed by NMR spectroscopy, and the results indicated that degradation did not take place. The determined CMCs were 0.08 mM for Tween 20, 0.06 mM for Tween 60 and 0.04 mM for a combined Tween 20, Tween 60 system.

1. Introduction

The use of suppositories as a dosage form has once again come into the foreground. Besides traditional antifebrile suppositories, suppositories containing probiotics are becoming more widespread. Ulcerative colitis and colorectal carcinoma are currently being diagnosed in rising numbers among children in Great Britain, and rectal drug administration is increasingly and more frequently resorted to in the pre- and postoperative therapy of such diseases [1–7].

Numerous studies that have focused on the liberation of drugs from suppositories containing surfactants from the aspect of pharmaceutical technology have clearly revealed that it is beneficial for a suppository base to have high hydroxyl group content. The usage of non-ionic surfactants is now suggested, but in lower amounts than those used in older recipes, which generally means lower than 3%, and preferably around 1% [8]. A high surfactant concentration may lead to the formation of micelles, which incorporate some of the API, impeding its release [9]. According to Ghorab et al. [10] optimum amount of Tween 60 is 5%, higher proportion than that up to 10% had lower effect on the release rate. Above 10% the release rate is decreased due to micelle formation. Surfactants not only enhance release of the drug form the suppository base but increase the permeability of the tissues surrounding the rectal lumen. Non-ionic surfactant Tween 20 showed outstanding effectiveness used in 5% amount combined with lipophilic vehicles [11].

One of the most characteristic properties of a surfactant is its CMC. In a specific solvent system, this is the concentration above which individual surfactant molecules spontaneously aggregate and form micelles through secondary interactions. Several methods are available for the determination of CMC. The most widely used techniques are the measurement of surface tension, conductance in the case of ionic surfactants, UV–vis spectrophotometry, NIR spectroscopy, turbidimetry and densitometry [12,13]. As the suppository base studied in the present work contained Tween 20 and Tween 60, in Table 1 we list the CMCs of these materials determined with different methods in previous studies.

In the present work, we focus on the role of non-ionic surfactants through the analysis of suppositories. In the case of generics, besides the technological formulation, the development of analytical methods is needed to acquire the data required for official

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Table 1
CMCs of Tween 20 and Tween 60.

Reference	Method	Temperature	Medium		
			H_2O	D_2O	Spec.[a]
Tween 20					
[14]	Surface tension	RT	0.0804	–	–
[15]	NIR	RT	0.08	0.12	70
[16]	Surface tension	RT	0.0488	–	–
[17]	Densitometry	24.88 °C	0.097	–	–
[18]	Surface tension	RT	0.059	–	–
[19]	Spectrophotometry of iodine absorbance	RT	0.055	–	–
	Shift in λ_{max}	RT	0.0557	–	–
	Surface tension	RT	0.0488	–	–
[20]	Surface tension	RT	0.048	–	–
[21]	Dye micellization	RT	0.042	–	–
	Surface tension	RT	0.011	–	–
Tween 60					
[17]	Densitometry	24.88 °C	0.068	–	–
[19]	Spectrophotometry of iodine absorbance	RT	0.0209	–	–
	Shift in λ_{max}	RT	0.0196	–	–
	Surface tension	RT	0.0206	–	–
[20]	Surface tension	RT	0.021	–	–
[21]	Dye micellization	RT	0.022	–	–
	Surface tension	RT	0.0055	–	–

[a] 1-Ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide $[EMIm]^+[Tf_2N]^-$.

registration. The sample preparation is an indispensable part of method development.

In the European Pharmacopoeia, there is no guidance as concerns general sample preparation for the assay of any dosage form; details are included only in the pharmaceutical technological procedures. The US Pharmacopoeia, on the other hand, contains monographs about pharmaceutical products, including suppositories [22]. However, micelle formation can cause problems during sample preparation. One plausible solution could be the use of microemulsion liquid chromatography (MELC) but this technique requires a complicated multicomponent mobile phase system [23]. Our present work is focused on extending the general knowledge relating to the analysis of suppositories, which can help those who choose rectal suppositories for drug delivery from both analytical and technological aspects; as such developments are conducted in parallel.

In Hungarian pharmaceutical practice, moulded suppositories are formulated predominantly with three suppository bases: hard fat (WitepsolW 35, adeps solidus in Hungarian practice), a hydrophilic base, which contains: macrogol 1540 and Span 20, as massa macrogoli. Third is adeps solidus compositus, which contains surfactants. In fact it is Witepsol W35 with Tween 20 and Tween 61. We will refer to this base in the present work as W35TT. A new HPLC method for the analysis of suppositories containing aminophenazone as model compound was previously developed and validated by our research group (unpublished data). In that work a new sample preparation method was elaborated for the recovery of the drug from hard fat or hydrophilic matrices. The method was intended to be applied to W35TT-based suppositories, too. However, in that case, with the well-established sample preparation method, the quantity of drug released was between 80% and 90%, which did not fulfil the requirements (95–105%) of the relevant ICH guideline. We present here a possible explanation and a solution for this phenomenon.

2. Materials and methods

2.1. Materials and instruments

The following materials were used: 4-dimethylaminoantipyrine (hereafter aminophenazone) (Sigma–Aldrich, St. Louis, MO, USA), paracetamol (Ph.Eur. 6.0, Phoenix Pharma Zrt., Hungary, Lot. No.: 1011204), methanol (Chromasolv for HPLC, Sigma–Aldrich, St. Louis, MO, USA), acetonitrile (VWR, Prolabo, Fontenay, France), sodium acetate (Reanal, Budapest, Hungary), acetic acid 96% (VWR, Prolabo, Fontenay-sous-Bois, France), sodium hydroxide (Reanal, Budapest, Hungary), sodium chloride (VWR, Prolabo, Leuven, Belgium), potassium dihydrogenphosphate (Spektrum 3D, Debrecen, Hungary) and potassium hydroxide (Reanal, Budapest, Hungary), Suppositorium antipyreticum pro parvulo FoNo VII. (Naturland Ltd., Hungary, Lot. No.: 1938-1112 and Parma Produkt Ltd., Hungary, Lot. No.: 1209-1106). Throughout the experiments, HPLC grade solvents were used. The aqueous solutions were prepared with triple distilled water. During the spectrophotometric measurements, methanol (VWR, Prolabo, Fontenay-sous-Bois, France), TWEEN® 20 (Sigma–Aldrich, St. Louis, MO, USA) and TWEEN® 60 (Sigma–Aldrich, St. Louis, MO, USA) were used.

The suppository bases applied were hard fat and W35TT (University Pharmacy, University of Szeged, Szeged, Hungary). Adeps solidus is officially included in Ph.Eur. as hard fat. W35TT is a special lipohydrophilic base, which is included officially in Fo.No. (Formulae Normales, the Hungarian collection of standard prescriptions.) It is a mixture of 95% (w/w) of hard fat, 2.5% (w/w) of Tween 20 and 2.5% (w/w) of Tween 61. In consequence of procurement issues relating to Tween 61, Tween 60 is nowadays used instead.

For eluent degassing and sample sonication a DLS 310-T DONAU-LAB-SONIC ultrasonic bath was used.

HPLC measurements were carried out on a Shimadzu Prominence UHPLC system (Shimadzu Corp., 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 achieved on a Hypersil ODS (C18) 150 mm × 4.6 mm, 5 μm column (Thermo Scientific, Keystone, UK). Data acquisition and peak integration were effected with LCSolution (Shimadzu Corp., Japan) chromatographic data acquisition and processing 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.

Table 2
Surfactant concentration ranges of the CMCs determination.

	Concentration (mM)												
Tween 60	0	0.03	0.04	0.05	0.06	0.08	0.09	0.1	0.2	0.3	0.5		
Tween 20	0	0.04	0.05	0.06	0.07	0.08	0.1	0.3	0.5				
Tween 20 and 60	0	0.001	0.005	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1	0.3	0.5

^{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 s, the acquisition time was 2.05 s and 8 transients have been collected into 32 K datapoints. The spectral processing included an exponential filtering with 0.3 Hz, zero-fillig to 64 K datapoints and a complex Fourier transformation. The data-collection and data-processing were carried out using Bruker XWIN-NMR 3.1 software.

2.2. Methods

2.2.1. Preparation of hard fat-based suppository containing aminophenazone as model compound for HPLC analysis

The suppository was weighed on an analytical balance, and was then placed into 15 ml of a mixture of methanol–water (50:50, v/v). The suppository was melted on a 40 °C water bath and the fatty phase was frozen by placing the vessel in ice. The liquid was filtered through moistened cotton to separate the frozen fat from the solution. The filtrate was collected in a 50 ml volumetric flask. The beaker was washed with once 15 ml and twice 5 ml of solvent and the washing solvent was combined with the solution in the volumetric flask, which was then made up to volume with the same solvent. 0.3 ml of stock sample solution obtained this way was diluted to 10 ml and filtered through a 0.45 μm pore size nylon membrane filter. The clear filtrate was injected into the HPLC system.

2.2.2. Preparation of W35TT-based suppository containing paracetamol as model compound for HPLC analysis

The suppository was weighed on an analytical balance, and was then placed into a beaker containing 15 ml mixture of methanol–acetonitrile–potassium dihydrogen phosphate (pH 6.3; 0.05 M) (50:5:45, v/v/v) (later: solvent), 1 ml 5 M sodium chloride and 2 ml 1 M sodium hydroxide. The suppository was melted on a 40 °C water bath. After that the beaker with the solution was placed into ultrasonic bath for 30 min. The following steps of sample preparation were the same as in Section 2.2.1 beginning with filtration.

2.2.3. Aminophenazone determination with HPLC

Aminophenazone content was determined with an HPLC method. The mobile phase was methanol–sodium acetate (pH 5.5; 0.05 M) (60:40, v/v). The pH of the sodium acetate buffer solution was set to 5.5 with acetic acid. The flow rate of the reversed-phase isocratic eluent was 1.5 ml/min and the run time was 5 min. The chromatographic column was thermostated at 30 °C. The chromatograms were recorded at 243 nm. The retention time of aminophenazone was 1.8 min.

2.2.4. Paracetamol determination with HPLC

The mobile phase during the quantitative determination of paracetamol was acetonitrile–potassium dihydrogen phosphate (pH 6.3; 0.05 M) (10:90, v/v). The buffer was prepared with potassium dihydrogen phosphate and the pH of the solution was adjusted to 6.3 with 1 M potassium hydroxide solution. The flow rate, the run time and the column temperature were the same as described in Section 2.2.3. The chromatograms were recorded at 241 nm. The retention time of aminophenazone was 2.3 min.

2.2.5. Active substance identification by NMR spectroscopy

The stability of the prepared samples was verified to make sure that the applied sample preparation method did not cause any degradation or decomposition of the active substance (aminophenazone or paracetamol) and that its structure remained unchanged throughout the experiment. The measurements were carried out at 25 °C.

The analytical sequence for both model compounds incorporated the measurement of a reference solution, which contained the appropriate compound in the solvent used during the chromatographic sample preparation, and the sample solution itself. The method of sample preparation for the HPLC measurements had to be altered slightly as the NMR studies required higher concentrations of the active substances. A 5-fold amount of aminophenazone or paracetamol (500 mg) was therefore dissolved in the appropriate solvent to 50 ml in the case of the reference solutions. The sample solution was prepared similarly to the reference solution, except that one W35TT blank suppository was dissolved in the solvent together with 1 ml of 1 M sodium hydroxide and 1 ml of 5 M sodium chloride solution. For the paracetamol measurements, 500 mg of substance was dissolved for the reference solution in 50 ml of solvent described in Section 2.2.2. The solution modelling the sample was prepared by dissolving 500 mg of paracetamol, and placing one blank W35TT suppository in the first 15 ml of solvent. Micelle demulsion was achieved according to Section 2.2.2 except the last dilution step. 10% (v/v) of D_2O was added to each sample before the NMR measurements.

2.2.6. CMC determination

We determined the CMCs of the components present in the aminophenazone-containing test suppositories in the applied solvent mixture: methanol–water (50:50, v/v). We used spectrophotometry for the measurement of the CMCs of the non-ionic surfactants Tween 20 and Tween 60. Three series of solutions were prepared with a constant amount of hard fat and increasing concentrations of the two surfactants, either separately or together. The concentrations used are listed in Table 2.

The concentration range for the combined surfactants was determined from the data for the initially characterized individual Tween 60 and the Tween 20. The highest concentration in the series was always 0.5 mM, which corresponds to that of both surfactants in the sample solution in the analytical method for suppositories containing W35TT.

The pseudoabsorbances (turbidity) of the series of solutions were determined at 550 nm at ambient temperature. The absorbances obtained in this way were plotted against surfactant concentrations. The linear regions of the plots were fitted by straight lines and the intersection of the straight lines gave the concentration of the CMC. The CMC can be identified as the breakpoint of the absorbance vs. concentration curve [24,25].

2.2.7. Dissolution test of hard fat and W35TT suppositories

Dissolution tests were carried out in a Hanson SR8+ dissolution tester equipped with basket apparatus. Commercially available

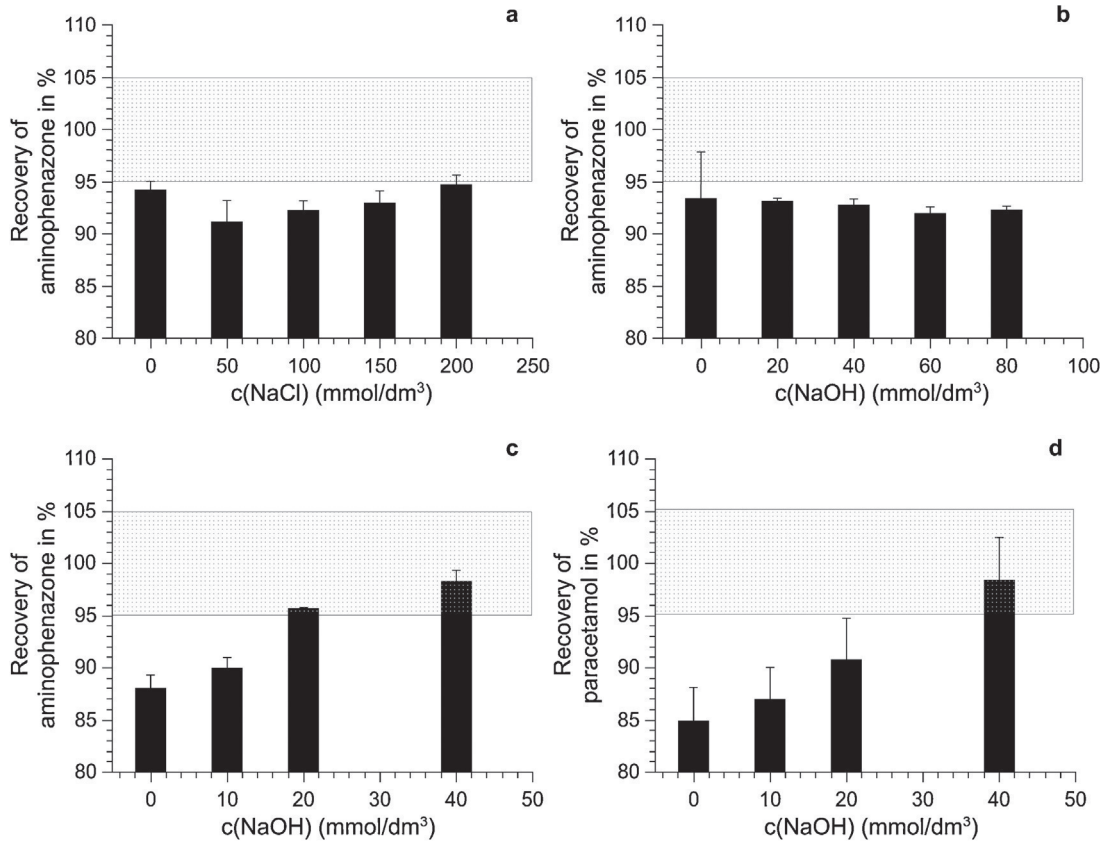

Fig. 1. Recovery of aminophenazone and paracetamol. (a) Effect of NaCl concentration. (b) Effect of pH. (c) and (d) Effects of pH at constant c(NaCl) = 100 mM. Vertical bars represent the mean of 3 independent measurement (n = 3), error bars show the standard deviation of the three data. Covariances between the independent variable (concentration) and the dependent variable (recovery) for plot a = 28.67; b = −13.47; c = 58.71 and d = 75.38.

suppositories prepared for children containing aminophenazone and either hard fat or W35TT were tested. Dissolution medium, potassium dihydrogen phosphate buffer (pH 7.5; 0.05 M), was thermostated to 37 °C. The agitation speed was 50 RPM. Samples were collected at 5, 10, 15, 30, 45, 60 and 120 min and tested by HPLC with the system described in Section 2.2.3.

3. Results and discussion

As W35TT is based on hard fat, it seemed reasonable to choose the sample preparation method described in Section 2.2.1 for hard fat as the starting point for the development. However, our expectations were not fulfilled; the recovery of the API from the W35TT base was only 88%. The surfactants formed micelles within the suppository base, which encapsulated some of the API. The sample preparation procedure described in Section 2.2.1 for hard fat was not suitable for quantitative release of the drug from the micelles for the analysis. This caused the difference in recovery between the two bases. In order to achieve a satisfactory release, the micelles had to be broken down to gain access to the trapped drug. It was presumed that salting-out might be a suitable method to solve this problem.

3.1. Effect of sodium chloride concentration on drug recovery

The procedure applied in the case of hard fat was amended with an additional step by adding sodium chloride solution in increasing concentrations to the first 15 ml of solvent in order to study the micelle-opening effect of salting-out. At all levels, two parallel samples were prepared. The sodium chloride concentrations applied were 0, 50, 100, 150 and 200 mM. HPLC analysis revealed that the recovery from all of the samples remained below 95%. Fig. 1 demonstrates that the increasing sodium chloride concentration did not correlate with the observed release. Calculated covariance between the concentrations and the recoveries suggest a slightly increasing relationship, which was possibly due to the minor CMC-decreasing effect of strong electrolytes [26,27]. The question may arise whether further increase of sodium chloride concentration could have improved the recovery sufficiently. On the other hand higher sodium chloride concentration would probably have caused the precipitation of the salt in the mobile phase as sodium chloride has solubility one order of magnitude lower in methanol than in water. Precipitation could damage the silica core of the column; therefore higher salt concentrations were not tested.

3.2. Effect of pH change on drug recovery

Micelle formation can be influenced by change of pH [28]. The API recovery was therefore studied in analytical samples containing increasing sodium hydroxide concentrations. The additional step in this case was the addition of 5 M sodium hydroxide solution to the initial 15 ml of extraction solvent during the melting of the suppository. Two parallel samples were prepared, in which the concentrations were 0, 20, 40, 60 and 80 mM. The HPLC analysis indicated that none of the recovery results reached the lower limit of the required range of 95–105% at any sodium hydroxide concentration. Similarly to the sodium chloride concentration study, these results did not exhibit any correlation with the increasing base concentration. The covariance of the series of data (see caption of Fig. 1) indicates a negative relationship. Therefore higher sodium hydroxide concentrations were not studied.

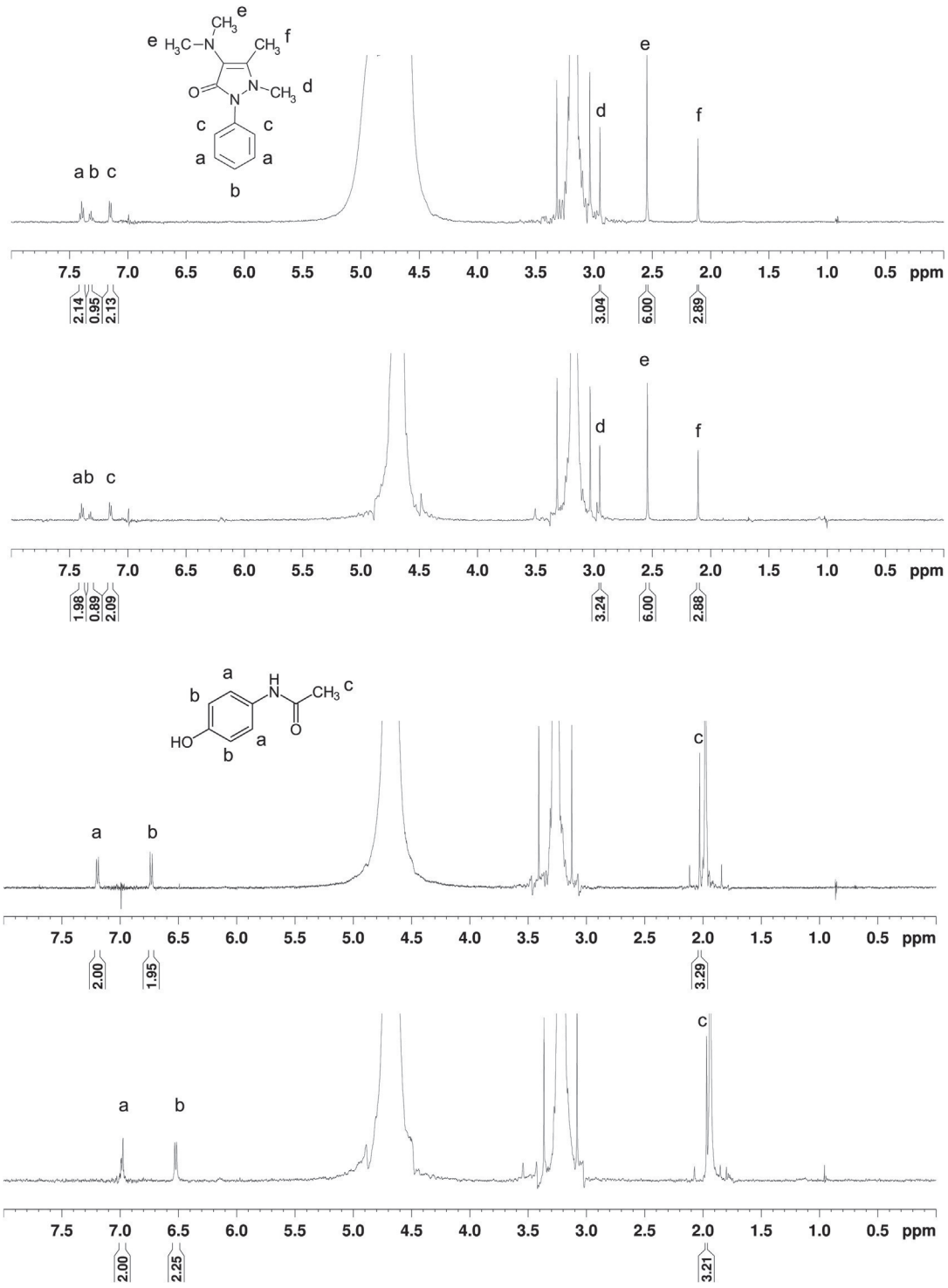

Fig. 2. [1]H NMR spectra of aminophenazone and paracetamol standards and samples. The signals marked with letters prove that no decomposition takes place in the sample solution treated with strong base. Peaks a and b of paracetamol are shifted to the right by 0.2 ppm due to the deprotonation of the OH and NH groups in the alkaline medium.

3.3. Effect of ultrasonic treatment on drug recovery

Micelles can be effectively destroyed by ultrasonic treatment. Due to the ineffectiveness of pH change and salting out, we applied 30 min ultrasound on 3 parallel preparations of suppositories. Average recovery was (92.6 ± 2.8)%. Due to the fact that during longer ultrasonic treatment the transfer medium is warming, longer agitation times were not applied. The ineffectiveness of the individual effects described in Sections 3.1–3.3 led us to study the combined effect of sodium chloride, sodium hydroxide and ultrasonic treatment simultaneously.

3.4. Combined effect of sodium chloride and sodium hydroxide on the drug recovery

The combination of sodium chloride and sodium hydroxide was tested together with an additional 30 min ultrasonic treatment. Constant, 100 mM sodium chloride and 0, 10, 20 or 40 mM sodium

Table 3
Calculation of CMCs from the data of fitted straight lines.

	Slope ± SD	Intercept ± SD	Intersect (mM)
Tween 20	0.4 ± 0.110 2.8 ± 0.485	0.24 ± 0.033 0.04 ± 0.027	0.084
Tween 60	0.52 ± 0.039 4.1 ± 1.022	0.12 ± 0.011 −0.096 ± 0.047	0.061
Tween 20 + Tween 60	2.1 ± 0.163 12.6 ± 2.083	0.75 ± 0.040 0.34 ± 0.032	0.040
Tween 20 + 60 with salt and base	0.07 ± 0.011 0.529 ± 0.008	1.99 ± 0.199 −0.09 ± 0.037	0.324

hydroxide were applied. The recovery results were favourable at 20 and 40 mM sodium hydroxide: 95.7% and 98.0%, respectively. Fig. 1 clearly reveals a correlation between the sodium hydroxide concentration and the recovery of the API when sodium chloride was present in the sample. The application of 100 mM sodium chloride and 40 mM sodium hydroxide was suggested.

This sample preparation method was also tested on paracetamol. Samples were prepared by the combined sodium chloride and sodium hydroxide addition method described in Section 2.2.2. The suppository was modelled by combining the blank hard fat suppository, Tween 20 and Tween 60 in the appropriate proportion and exactly weighed 100 mg of paracetamol. The sodium hydroxide concentration was set to 0, 10, 20 or 40 mM during the experiment. The recoveries demonstrated by HPLC analysis are presented in Fig. 1 plot d. In the case of paracetamol, 40 mM sodium hydroxide was required to liberate the API entirely. The average recovery was found to be 98.4%.

Covariance values for plots c and d of Fig. 1 express a strong positive relationship between the increase of the hydroxide concentration and the change of the recoveries. These values are significantly higher than those determined for plots a and b of Fig. 1.

3.5. Stability of the drugs during sample preparation

In both cases, the drugs were subjected to treatment with strong base. Sodium hydroxide is suggested to be used between 20 and 40 mM, but it must be ensured that the drugs do not suffer degradation during the treatment. The first check was during the HPLC runs, where the spectra of the samples and the pure substances proved to be identical. The retention times were also found to be similar. Finally, the ^{1}H NMR spectra of the pure standards and the treated samples were also recorded. In the assigned spectra of paracetamol, the signals of the OH and NH protons are not visible due to the fast exchange with water. It is clearly seen in the paracetamol sample spectrum that, due to the deprotonation of the OH and NH groups with the increase of pH, the chemical shift of the aromatic protons decreases by 0.2 ppm. In the case of aminophenazone, no deprotonation takes place, and thus there is no change in the chemical shifts.

The overlaid spectra showed no significant difference, i.e. no new peaks were detected, which confirms that the samples remained intact during the sample preparation (Fig. 2).

3.6. CMCs of Tween 20 and Tween 60

The CMCs of Tween 20 and Tween 60 were determined in the presence of hard fat by measuring the turbidity of solutions with different surfactant concentrations at 550 nm. Turbidity was plotted against surfactant concentration. Straight lines can be fitted to each series of points, the intersect of which gives the CMC in the specific solvent system. This intersection can also be identified as the breakpoint of the curve. The parameters of the fitted straight lines and their intersects are presented in Table 3. In methanol–water (50:50, v/v) as solvent the CMC of Tween 20 was 0.084 mM, that of Tween 60 was 0.061 mM, and that of the two surfactants together was 0.040 mM.

These results are in good agreement with the trends reported in previous studies. The slight differences from the literature data can be attributed to the different measurement techniques and the different solvents [15,18]. The solvent in this case, an equivolume mixture of water and methanol, could increase the CMC. Short-chain alcohols such as methanol or ethanol are known to decrease the hydration of the ethyleneoxide chains of non-ionic surfactants, so the observed trend in the CMC increase is in good agreement with this explanation [29]. The tendencies earlier found for different Tween substances can be observed in our results too: the CMC of Tween 20 is higher than that of Tween 60. Moreover, the two surfactants together give a significantly lower CMC, which is also characteristic [14]. It is obvious that the applied surfactant concentration in the suppository base is well above the determined CMC, which explains the micelle formation and the decreased recovery of drug as compared with the "pure" hard fat. Although the application of surfactants in the suppository base has a beneficial effect on the formulation properties of the material and on the drug release in the body, the amounts of surfactants must be optimized to avoid the analytical pitfall of failing the recovery study. Too high a surfactant concentration will always pose a potential risk of analytical error.

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 whether this phenomenon would cause homogeneity problems at surfactant concentrations higher than the CMC in suppositories produced in the industrial technology, which is based mainly on moulding techniques for large quantities. Our research group is conducting further studies to find answers for these questions.

The complex sample preparation method, which was tested with two different model compounds, is rapid and effective in the case of suppository bases containing surfactants to enhance the liberation of the encapsulated drugs. The success of the development can be explained through the micelles losing their colloidal stability due to the loss of electrostatic interactions, which is caused by the addition of electrolytes. The mechanism is similar to that in formulations with targeted drug release. This hypothesis was proven by determining the CMC of Tween 20 and Tween 60 in a medium where final sodium chloride concentration was 100 mM and sodium hydroxide concentration was 40 mM. The results can be seen in Fig. 3. The CMC of the combined surfactants is about 5 times higher in the presence of salt and base than in the absence of them. The applied salt and base concentration combined with the ultrasonic treatment was sufficient to raise the CMC of the system so that the surfactants within the suppository could not assemble into micelles entrapping part of the API.

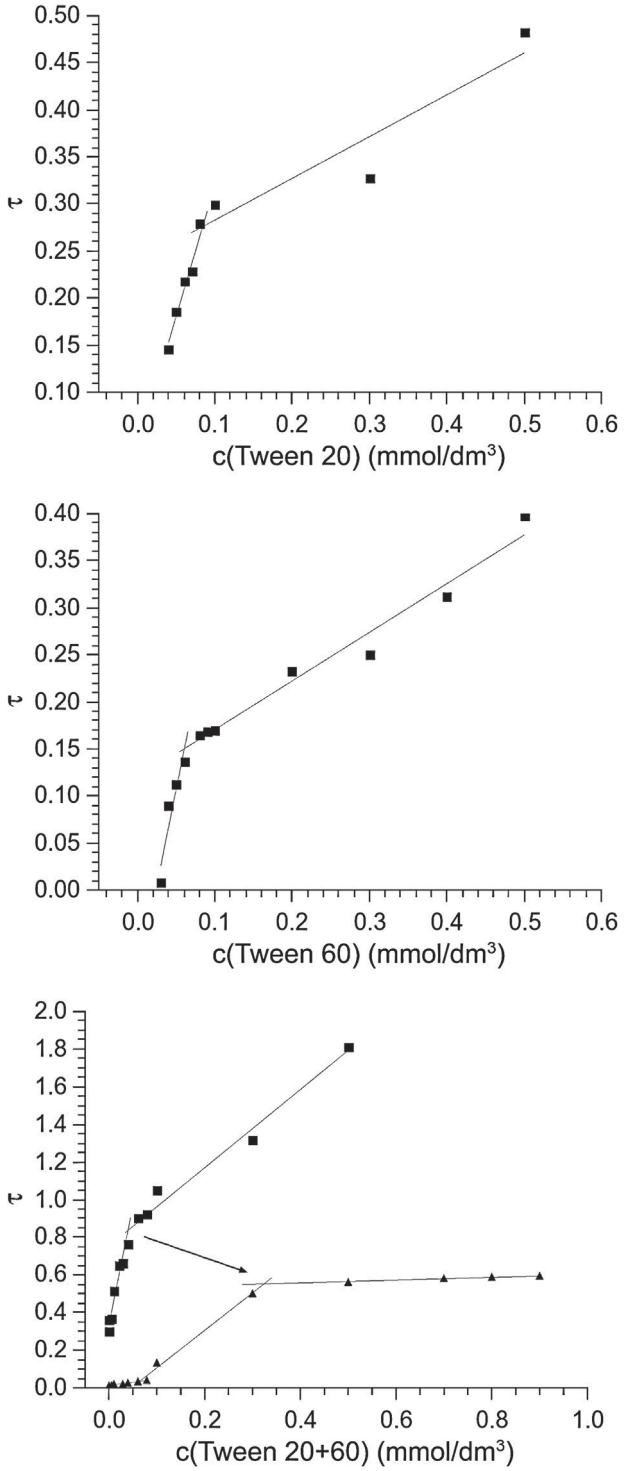

Fig. 3. Turbidimetric plots for the determination of the CMCs of Tween 20 and Tween 60.

3.7. Dissolution profile of different base suppositories

Six dosage units were tested from both hard fat and W35TT containing suppositories according to the parameters described in Section 2.2.7. It can be seen in Fig. 4 that dissolution rate from solid fat based suppositories is slower in the time range critical for the in vivo effect and until 120 min only 70% of the drug is released. W35TT based suppositories saw saturation from 60 min (88.9 ± 10.9%). On the other hand it should be noted that during these experiments the volume of dissolution medium was 900 ml where the concentration of Tween 20 is 0.0244 mM and concentration of Tween 60 is 0.0228 mM, which is significantly lower than in case of our analytical samples. Application of such large volumes for sample preparation to avoid micelle formation is not recommended in routine laboratories. Furthermore it is worth for consideration that dissolution rate in large volumes is influenced by surfactants favourably because their concentration fall below their CMC, but this for sure cannot model the physiological conditions where the volume of rectal fluid is only 2–3 ml. In such a small volume the surfactant concentration will be definitely higher than those in our analytical samples and will fall above the CMC in every case. If 2.5 ml rectal fluid volume is hypothesized concentration of Tween 20 will be 9.76 mM and concentration of Tween 60 will be 9.12 mM accounting for even higher extent of micelle formation.

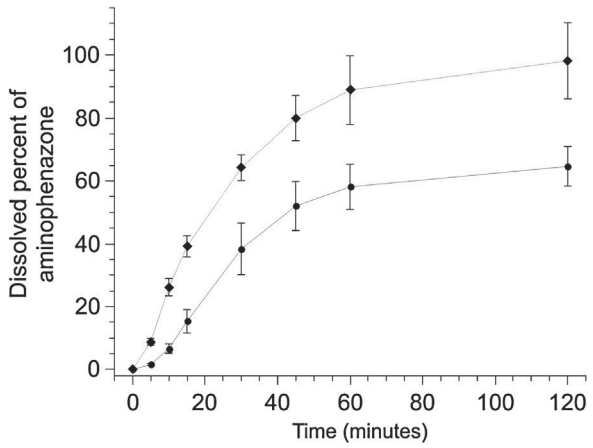

Fig. 4. Dissolution profiles of aminophenazone containing hard fat (●) and W35TT (♦) suppositories.

4. Conclusions

We have developed an adequate sample preparation method for Tween 20 and Tween 60 containing hard fat based suppositories. We have proved that micelles are being formed in the sample solution and successfully destabilized them by applying 100 mM sodium chloride and 40 mM sodium hydroxide concentration and 30 min ultrasonic 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 as well.

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ORIGINAL ARTICLE

Dosage uniformity problems which occur due to technological errors in extemporaneously prepared suppositories in hospitals and pharmacies

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KEYWORDS

Quality control;
Rectal administration;
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Cerimetric titration;
Clinical pharmacy practice

Abstract The availability of suppositories in Hungary, especially in clinical pharmacy practice, is usually provided by extemporaneous preparations. Due to the known advantages of rectal drug administration, its benefits are frequently utilized in pediatrics. However, errors during the extemporaneous manufacturing process can lead to non-homogenous drug distribution within the dosage units. To determine the root cause of these errors and provide corrective actions, we studied suppository samples prepared with exactly known errors using both cerimetric titration and HPLC technique. Our results show that the most frequent technological error occurs when the pharmacist fails to use the correct displacement factor in the calculations which could lead to a 4.6% increase/decrease in the assay in individual dosage units. The second most important source of error can occur when the molding excess is calculated solely for the suppository base. This can further dilute the final suppository drug concentration causing the assay to be as low as 80%. As a conclusion we emphasize that the application of predetermined displacement factors in calculations for the formu-

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lation of suppositories is highly important, which enables the pharmacist to produce a final product containing exactly the determined dose of an active substance despite the different densities of the components.

1. Introduction

Drugs used for rectal administration are frequently supplied by independent pharmacies and especially clinical pharmacies. This route of administration is very important in pediatrics. Pharmaceuticals used for the treatment of fever, pain, spasms, asthmatic symptoms and vomiting can be administrated rectally (Abd-el-Maeboud et al., 1991; Dahl et al., 2000; Fumoleau et al., 1997; Kauss et al., 2012; Okabayashi et al., 2012; Richter et al., 2012; Sabchareon et al., 1998; Shiohira et al., 2009; Tinner et al., 2013). Approximately 80% of the suppositories used in Central Europe are produced extemporaneously using the molding technique. In clinical pharmacies quantities of 100–300 and in independent pharmacies 10–12 suppositories are generally molded as one batch. Suspension suppositories, in particular, are formulated with a solid fatty vehicle (''e.g.'' Witepsol 35) or a combination of this suppository base with surfactants (Rowe et al., 2003). The core of this technology is the dispersion of the finely powdered drug with the molten suppository base. After which, the suspension is molded under continuous stirring. Fatty suppository bases have very low viscosities, which decrease still further with an increase in temperature, causing rapid sedimentation of the suspended particles and leading to a non-homogeneous product. When the liquid mass is molded at around the solidification point, solidification occurs immediately as the mass enters the mold, making further additions of the base and drug impossible. In the calculation of the suppository base weight, the following formula must be applied (Eq. (1)):

$$T_m = E - \sum_{i=1}^{n} f_i \cdot s_i \quad (1)$$

where T_m is the suppository base to be weighed, E is the calibration constant of the mold, f_i is the displacement factor of the ith component and s_i is the weight of the ith component. During the calculation of a correct formula, it is not sufficient to subtract the weight of the solid components from the final weight of the suppository to obtain the required amount of the suppository base. We must also know the value of E for the specific mold and specific suppository base, which can be determined through independent measurements. Ten suppositories are usually prepared with the mold, using the pure base, and after cooling they are weighed and the average suppository weight is calculated. This average value will be used as the calibration constant for the mold for any specific base. Since the density of the active ingredient (hereinafter referred to as ''API'') incorporated into the suppository base can differ from that of the base itself, the displacement factor (f) is required to compensate for the difference in densities. The value of f, which shows how much base will be displaced by a unit weight of an API, can be calculated from the following equation (Eq. (2)):

$$f = \frac{100 \cdot (E - G)}{G \cdot x} + 1 \quad (2)$$

where E is the weight of the blank suppository containing only base, G is the weight of the suppository containing an API in a known concentration, and x is the API content of the suppository in weight percentage.

If the pharmacist fails to carry out the steps in strict accordance with these rules, significant deviations will be observed in the results for the homogeneity of the batch and in the total drug content of the batch.In this research we report on an investigation of samples prepared in pharmacies, with a special emphasis on the homogeneity and the total API content of the batches. The circumstances of the preparation of the suppositories were known in all cases and are presented. In pharmacies, the f-values of the most frequent APIs in the most common bases are not generally available. According to a good manufacturing practice pharmacists apply the participle of overage during the calculation of the batch composition, but an incorrect calculation for the amount of vehicle required and other technological errors may lead to serious deviations in the final dosage for the individual suppositories (Allen, 2007; Miseta and Soós-Csányi, 2011; Rácz and Selmeczi, 1991).

2. Materials and methods

2.1. Materials

HPLC grade solvents and triple-distilled water were used during the chromatographic measurements. For the preparation of the HPLC mobile phases and sample preparation solvents, the following materials were used: 4-dimethylaminoantipyrine (Sigma–Aldrich, St. Louis, MO, USA), methanol (Chromasolv for HPLC, Sigma–Aldrich, St. Louis, MO, USA), sodium acetate (Reanal, Budapest, Hungary), acetic acid 96% (Molar Chemicals, Budapest, Hungary), sodium hydroxide (Reanal, Budapest, Hungary) and sodium chloride (VWR, Prolabo, Leuven, Belgium). Volumetric solutions for the cerimetric titrations were prepared with the following materials: cerium(IV) sulfate tetrahydrate (Panreac, Barcelona, Spain), sulfuric acid 96% (Farmitalia Carlo Erba, Milano, Italy) and ferroin-solution, 1/40 M (Reanal, Budapest, Hungary).

Commercially-made suppositories were used during the comparisons for the analytical methods. The reference product was *Suppositorium antipyreticum pro parvulo* FoNo VII. Naturland (Naturland Magyarország Kft., Budapest, Hungary), which contained 150 mg of aminophenazone per suppository in a solid fatty suppository base. One box contains six suppositories (Paál, 2003).

Samples were also prepared in regular pharmacies by the molding technique, according to the following procedure. Ten suppositories were obtained from 15 independent pharmacies with a labeled claim of 100 mg of aminophenazone in each suppository. The choice of vehicle for the suppository was left to the responsibility of the pharmacist at the site. Practically all of the samples were prepared with a solid fatty base. In each

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case, predetermined technological errors (known to us) were made during the sample preparation.

2.2. Test methods

2.2.1. Cerimetric titration

The basis of the drug content determination is the use of a cerimetric redox titration method (Rózsa, 1953), During this assay the nascent oxygen evolved from the reaction of Ce(IV) with water oxidizes aminophenazone. The end-point of the titration is observed by the change in color of ferroin added as the indicator. During the sample preparation, whether from the commercially prepared suppository or the extemporaneously prepared ones, one suppository is melted over a 40 °C water bath and 3 replicate samples of 0.20–0.30 g are weighed from the molten mass into titration flasks. A 10.0 ml portion of 15% sulfuric acid is added to each sample and the mixture is heated to 40 °C to extract the API from the suppository base. The mixture is then cooled to room temperature. A 15 ml portion of distilled water is added, and after mixing, one drop of ferroin as indicator is added. This is then titrated with 0.05 M cerium(IV) sulfate volumetric solution until the color of the solution changes from orange to green and remains green for at least 1 min (Paulenova et al., 2002; Townshend, 2005).

2.2.2. Assay of aminophenazone by HPLC

HPLC measurements were carried out on a Shimadzu Prominence UHPLC system (Shimadzu Corp., Kyoto, Japan) equipped with an LC-20AD pump, a four 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. Sample injection was performed with a Rheodyne six port manual injector valve fitted with a 20 µl sample loop. Separation was achieved on a Hypersil ODS (C18) 150 × 4.6 mm, 5 µm column (Thermo Scientific, Keystone, UK). Data acquisition and peak integration were carried out with the LC Solution (Shimadzu Corp., Kyoto, Japan) chromatographic data acquisition and processing software.

The mobile phase was methanol:sodium acetate (pH 5.5; 0.05 M) (60:40, v/v). The pH of the sodium acetate buffer solution was adjusted to the desired value with acetic acid. The flow rate of the reversed-phase isocratic eluent was 1.5 ml/min and the run time was 5 min. The chromatographic column was thermostated at 30 °C throughout the separation. The chromatograms were recorded at 243 nm. The retention time of aminophenazone was found to be 1.8 min. The applied method was validated. The samples were prepared with a technique elaborated previously by our research group. The exactly weighed suppository was melted over a 40 °C water bath in a 50:50 (v/v) methanol:water mixture. After identification of the suppository base on the basis of its physicochemical properties, the base was separated from the solution if necessary. The sample solution was then filtered through a 0.45 µm pore size nylon membrane filter. The solution was injected onto the HPLC through the Rheodyne injector valve.

3. Results and discussion

3.1. Comparison of the cerimetric titration and the HPLC method

We compared the two analytical methods by measuring two sets of six commercially-made suppositories from the same batch having the exact lot number. The individual assays for the suppositories were carried out by either the volumetric or the chromatographic technique. All of the final results (the averages of the three replicates for each of the six suppositories in the case of the titrimetric method, and the individual suppository assay value in the case of the chromatographic determination) fell within the range 95–105%, which conforms to the strictest requirements of the European Pharmacopoeia. The individual content data are presented in Table 1.

It should be noted that the volumetric results exhibit a larger standard deviation than that of the chromatographic results, but the preparation of samples for titration (one total of 18 replicate samples prepared from the molten suppository) involves a higher level of uncertainty, and this can therefore be considered acceptable. A comparison of the two methods reveals that both can be used for the analysis of suppositories containing aminophenazone.

3.2. Dosage uniformity study of extemporaneously prepared suppositories

We additionally studied pediatric suppositories with the composition described in Section 2.1. All samples were from different Hungarian independent pharmacies, and each sample consisted of 10 individual suppositories. The manufacturing technology for each of the samples was known to us with the pharmacists committing intentional technological errors during the preparation of the suppositories. The extemporane-

Table 1 Assay results for factory-produced suppository samples, measured by cerimetric titration or the HPLC method.

Sample	Cerimetric titration				Sample	HPLC
	Replicates%			Average cont.%		Content%
S1	101.6	107.2	103.5	104.1	S7	103.9
S2	98.9	99.3	97.5	98.5	S8	104.9
S3	106.3	100.7	100.3	102.4	S9	102.8
S4	96.1	100.7	97.1	98.0	S10	102.3
S5	100.9	110.4	102.1	104.5	S11	103.9
S6	103.6	104.0	104.7	104.1	S12	104.6
Average				101.9		103.7
SD				2.93		1.01

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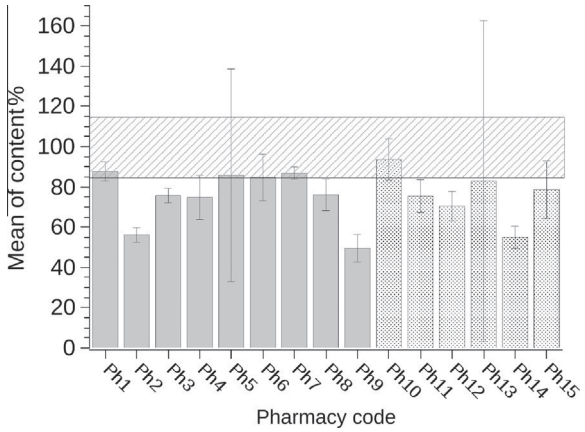

Figure 1 Mean API content for the samples, with the standard deviations. An API content in the interval 85–115% is satisfactory. Samples Ph1–Ph9: HPLC results; Samples Ph10–Ph15: cerimetric titration results.

ously prepared samples (containing 10 suppositories) from pharmacies Ph1–Ph9 were measured with the HPLC technique described in Section 2.2.2. The extemporaneously prepared samples (containing 10 suppositories) from pharmacies Ph10–Ph15 were tested for dosage uniformity with the titrimetric method presented in Section 2.2.1. All of the findings (see Fig. 1) were compared on the basis of the results given in Section 3.1. It can be stated that two samples conformed to the specifications of Ph. Eur. 7.8, "i.e." the individual assay values fell within the range 85–115% at level 1, and one further sample would probably have conformed to the level 2 specification of 75–125% (Ph Eur 7.8). For the remaining samples, generally either lower individual assay results or (in 2 samples) significant non-homogeneity was found, which are justified by standard deviation values visible in Table 2.

3.3. Effects of f-value on the assay results

The possible consequences of the most common errors can be illustrated utilizing a theoretical example. If the pharmacist produced suppositories on the basis of the following parameters: $E = 1.7$ g; $f = 0.78$ and $s = 0.1$ g then, according to Eq. (1), $T_m = 15.22$ g for the 10 suppositories. If f-value is not applied, but only the weight of the API is subtracted from the value of E, then we have $T_m = 16.0$, which will result in an assay which is 4.6% lower than the required value. If this error is superimposed with the one when the calculated molding excess is taken solely from the suppository base (taking the required base for 12 dosage units instead of 10) then the concentration of one suppository is diluted even further, to 80.3% of the intended theoretical value.

3.4. Effects of stirring on the homogeneity and total assay of the samples

The suppository mass can be well homogenized by choosing an appropriate rate of manual stirring or machine-based mixing. Stirring during the molding process can help avoid the sedimentation of the API in the container. Too slow a stirring rate is not effective, however too fast a stirring rate may also lead to errors: since air bubbles may be formed in the mass, which will decrease the weight of the suppositories. Foam can be formed from the surfactant-containing bases, or shearing forces may appear, which decrease the viscosity of the suppository mass by rheodestruction, causing the rapid sedimentation of the API. The ideal machine stirring speed for fat-based suppositories is recommended as 150 rpm.

The results demonstrate that the stirring technique for extemporaneously prepared pharmacist suppositories before and during the molding procedure was appropriate, with the exception of a small number of serious cases. Those samples can be considered homogeneous which gave SD $\leqslant$ 10 with respect to the individual suppository assays. A larger deviation can originate from the lack of stirring during molding, as may be seen for samples 5, 7 and 16. Extremely large deviations result when both thorough homogenization and stirring during the molding process are omitted, which maybe observed for samples 6 and 13. If (*f*) is not applied and the suppository base is used in an excess amount, the assays for the samples will fall below the lower limit of acceptance. If the molten mass is not stirred during molding process, the decrease in the API content becomes more serious since the suspended material is sedimented, and the mold will contain an API – which is depleted from the mixture.

4. Conclusions

The results for pediatric 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 Ph. Eur. 7.8. Since extemporaneously prepared suppository preparations are frequently compounded and supplied in central European clinical pharmaceutical practice because of the low costs involved, we 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 our knowledge the paucity of this information prohibits the preparation of the "right" dose for the "right" patient and may even do harm. Calibration of

Table 2 Average assay results on the samples and standard deviations in the homogeneity study. Ph in the sample raw stands for pharmacy.

Sample	Ph1	Ph2	Ph3	Ph4	Ph5	Ph6	Ph7	Ph8	Ph9	Ph10	Ph11	Ph12	Ph13	Ph14	Ph15
Average%	87.7	56.1	75.6	74.8	85.8	84.7	86.9	76.2	49.5	93.7	75.5	70.4	82.9	54.9	78.6
SD	4.8	3.6	3.6	11.1	52.8	11.6	3.0	7.8	7.0	10.2	8.0	7.4	79.7	5.5	14.3

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the mold and the determination of the *f*-value for these basic common suppository bases can be accomplished very simply.

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III.

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Article

Simultaneous Quantification of Paracetamol, Acetylsalicylic Acid and Papaverine with a Validated HPLC Method

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Combined drug products have the advantages of better patient compliance and possible synergic effects. The simultaneous application of several active ingredients at a time is therefore frequently chosen. However, the quantitative analysis of such medicines can be challenging. The aim of this study is to provide a validated method for the investigation of a multidose packed oral powder that contained acetylsalicylic acid, paracetamol and papaverine-HCl. Reversed-phase high-pressure liquid chromatography was used. The Agilent Zorbax SB-C18 column was found to be the most suitable of the three different stationary phases tested for the separation of the components of this sample. The key parameters in the method development (apart from the nature of the column) were the pH of the aqueous phase (set to 3.4) and the ratio of the organic (acetonitrile) and the aqueous (25 mM phosphate buffer) phases, which was varied from 7:93 (v/v) to 25:75 (v/v) in a linear gradient, preceded by an initial hold. The method was validated: linearity, precision (repeatability and intermediate precision), accuracy, specificity and robustness were all tested, and the results met the ICH guidelines.

Introduction

A number of drug products are available on the market for the treatment of smooth muscle spasm, e.g., in the biliary, renal and intestinal tracts. For the mitigation of acute renal or gastrointestinal pain, the primary drug of choice is a nonsteroidal anti-inflammatory drug (NSAID) such as paracetamol, acetylsalicylic acid (ASA) or ibuprofen (1). Such conditions are frequently treated with combined products, which contain a smooth muscle antispasmodic together with one or more NSAID painkiller(s) (2). The combination of papaverine hydrochloride (papaverine) and ibuprofen or indometacin is nowadays commonly used, especially for the treatment of dysmenorrhea. As regards the administration of papaverine, the research focus has shifted in recent years from the gastrointestinal tract to the coronary arteries (3) and the therapy of an erectile dysfunction (4–6). Nevertheless, in pharmaceutical practice, papaverine is still commonly prescribed as an antispasmodic to relieve gastrointestinal and menstrual spasms.

When a rapid effect is desired, the active pharmaceutical ingredients (APIs) can be applied in powder dosage form without excipients. Analgesic drugs are often formulated as multidose packed oral powders. An oral powder as a pharmaceutical dosage form containing solid ingredients, including one or more APIs with or without excipients. It is generally administered in or with water or another suitable liquid. It may also be swallowed directly (7).

Many authors have described the simultaneous determination of paracetamol and ASA in various pharmaceutical dosage forms and also in blood or urine samples (8–18), but the available literature on the HPLC analysis of papaverine is quite limited. Mostly, the presence of papaverine together with opiates has been studied (19–27) and many findings are available as concerns its identification in blood samples from opiate drug users (20, 23, 24, 26, 27). Its UV–vis detection in chromatographic methods is very rare. The mobile phase compositions applied are often complex, containing multiple organic modifiers, which are beneficial from the aspect of papaverine, but do not facilitate the analysis of samples containing paracetamol and ASA too. The NSAID components of the mixtures would elute within the void or coelute if the method suitable for paracetamol analysis were applied. Despite a thorough search, we have found no hits for the determination of paracetamol, papaverine and ASA with a single analytical method, and conclude that the simultaneous RP-HPLC analysis of these three components has not been previously published.

Experimental

Materials and instruments

The following materials were used in our studies: paracetamol (Ph. Eur. 6.0, Phoenix Pharma Plc., Hungary, Lot No.: 1011204), papaverine-HCl (Molekula, Shaftesbury, UK), ASA (Ph. Eur. 6.0, University Pharmacy, University of Szeged, Szeged, Hungary), methanol (Chromasolv for HPLC, Sigma-Aldrich, St. Louis, MO, USA), acetonitrile (ACN) (VWR, Prolabo, Fontenay-Sous-Bois, France), sulfuric acid 96% (Analyticals Carlo Erba, Milano, Italy), potassium dihydrogenphosphate (SPEKTRUM-3D, Debrecen, Hungary) and potassium hydroxide (Reanal, Budapest, Hungary). Throughout the experiments, HPLC grade solvents were used. The solvents and the aqueous solutions were prepared with triple distilled water.

HPLC measurements were carried out on a Shimadzu Prominence UHPLC system (Shimadzu Corp., Kyoto, Japan) equipped with an LC-20AD pump, a four-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 six-port manual injector valve fitted with a 20-μL sample loop. During the method development, separation was studied on a Hypersil ODS (C18) 150 × 4.6 mm, 5 μm column (Thermo Scientific, Keystone, UK), a Luna C18(2), 150 × 4.6 mm, 3 μm column (Phenomenex, Torrance, CA, USA) and a Zorbax SB-C18 150 × 4.6 mm, 3.5 μm column (Agilent, Santa Clara, CA, USA). 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 LCSolution and Microsoft Office Excel 2007 software. The log *D* vs. pH function was predicted with Pallas intelligent chromatographic software (28). The samples were filtered through a 0.45-μm pore size nylon membrane filter (Millipore Ireland Ltd., Tullagreen, Carrigthwohill, Ireland) before the injection.

Sample preparation

For the stock solution, 48.0 mg powder (17.0 mg paracetamol, 26.0 mg ASA and 5.0 mg papaverine in a homogeneous mixture) was weighed with analytical precision into a 50.0-mL volumetric flask, dissolved and made up to volume with the solvent, phosphate buffer (25 mM, pH 3.43): ACN (85:15, v/v). During the preparation, the sample was heated to 40°C, this step being required for the complete dissolution of ASA, which has low solubility (slightly soluble according to Ph. Eur.) in water. For the working sample solution, 3.0 mL stock solution was diluted to 10.0 mL and filtered through a 0.45-μm Millipore syringe filter before injection.

Results

Method development strategy

As the first step of the chromatographic method development, the properties of the drugs which may influence the separation were determined. Particularly, the separation of papaverine and ASA can be difficult to achieve in view of the specific p*K* values and the log *D* versus pH curves (Figure 1), which were calculated with Pallas chromatographic software. The pH of the applied aqueous mobile phase is one of the key parameters that affects the separation. The range between 2 and 8 is optimum from the aspect of the stationary phase, but the range between 1 and 6 is not appropriate for the separation of papaverine, which contains one basic nitrogen with a p*K* in the upper part of the range. In the pH interval 3–8, the ratio of the dissociated and undissociated forms of ASA changes. At pH >6 (which is beneficial for papaverine separation), ASA peak splitting was observed.

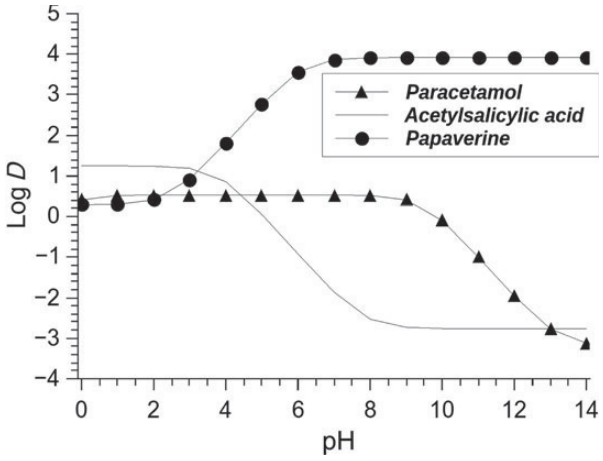

Figure 1. Log *D* versus pH curves of paracetamol, ASA and papaverine.

In light of the above findings, the most challenging task was to find the most appropriate combination of the boundary conditions, where the overall negative influence on the separation and elution of the analytes is least. pH 3.4 ± 0.05 was found to be a reasonable compromise for the pH of the aqueous phase. An assay of papaverine alone was reported in the application database of Agilent, which involved a similar pH in the aqueous mobile phase (http://www.chem.agilent.com/Library/applications/59681112.pdf). In this method, the aqueous eluent contained 25 mM potassium dihydrogenphosphate, but sulfuric acid was used to adjust the pH so as not to increase the phosphate concentration.

It can be seen in Figure 1 that at pH 3.4 paracetamol and most of the ASA are in an undissociated form. The basic papaverine is at the beginning of the transient section of the equilibrium, which can be observed between pH 3 and 6 in the log *D* curve. The ratio of the organic modifier of the mobile phase, ACN, was linearly increased from 7 to 80% during the initial 16 min of the run, and was then kept constant for 4 min. Between 20 and 22 min, the ratio of the organic modifier was linearly decreased to the initial level, at which it was held constant during the remainder of the run, to 25 min. A 1:1 (v/v) mixture of methanol and the mobile phase was suggested as solvent in the literature method. The flow rate of the mobile phase was 1.5 mL/min and the separation was achieved on a Hypersil ODS column at 60°C. The results of the runs under the above-described conditions can be seen in chromatogram (a) in Figure 2, where paracetamol and ASA were co-eluted. An initial isocratic hold was therefore inserted into the method before the gradient for the resolution of the co-elution, because the lower organic content selectively increased the retention times of the peaks, removing them from the void. In the new method, we applied a constant 7% ACN section during the initial 2 min, followed by a similar gradient as described above. At this point, it became obvious that the hydrophobicity of the stationary phase was too low and the retention of the basic papaverine was too high, so that it could not be eluted with acceptable peak shape within a reasonable time, although the separation of paracetamol and ASA was ideal.

For optimization of the peak shape, an alternative column had to be used. Two columns with different selectivity and with higher hydrophobicity than that of the Hypersil ODS column were chosen on the basis of the data to be found in the comparative column selectivity database of Waters (http://www.waters.com/waters/promotionDetail.htm?id=10048475&locale=en_US) the Luna C18(2) and the Zorbax SB-C18 stationary phases. It is clear from chromatogram (b) in Figure 2 that a hydrophobicity increase of <1 order of magnitude led to the successful elution of papaverine. This latter method resulted in the co-elution of ASA and papaverine on both columns. In order to resolve the peaks, the can content at the end of the gradient and in the second isocratic section had to be decreased from 80 to 25%.

This modification resulted in suitable peak separation for all three analytes on both Luna C18(2) and Zorbax SB-C18. ASA and papaverine were eluted with higher resolution on the more selective Zorbax SB-C18 column. The retention parameters of the separated peaks on the three different columns are presented in Table I. It is clear that the Hypersil ODS column was not suitable for the simultaneous separation of the three components,

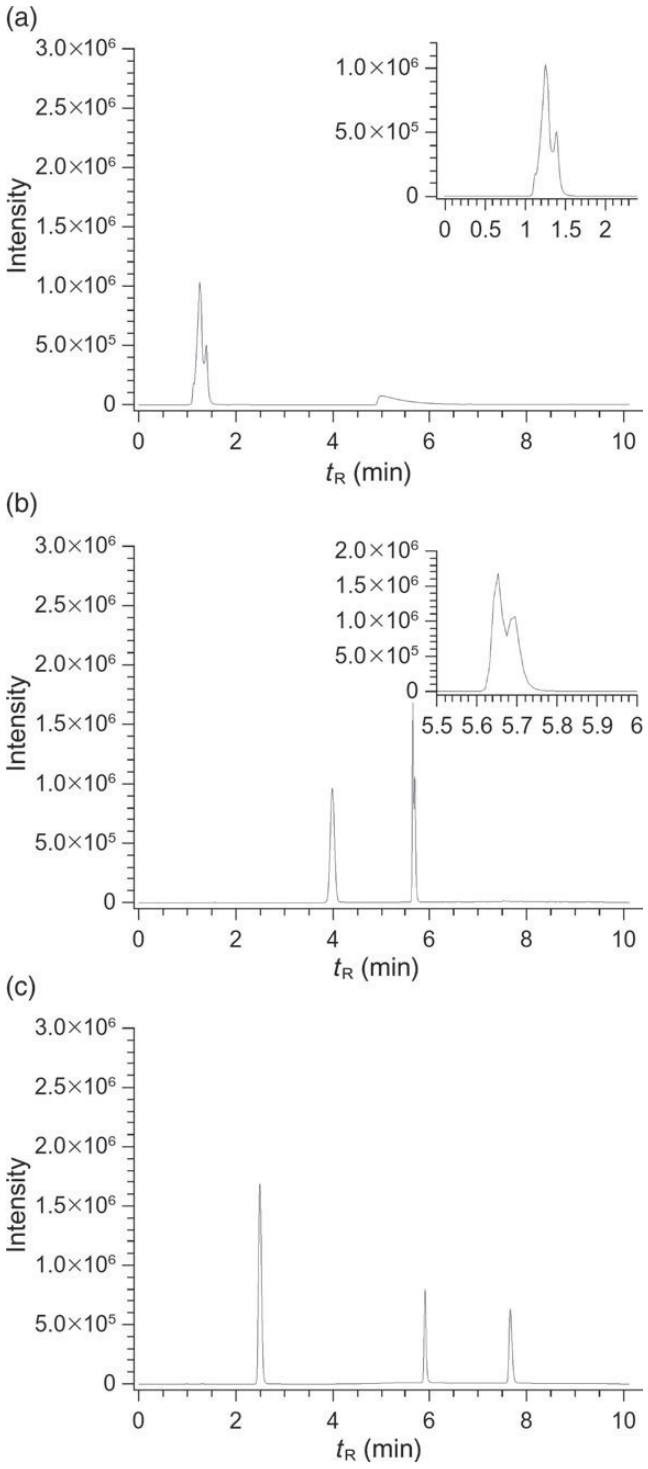

Figure 2. Chromatograms obtained on the Hypersil ODS (a), Luna C18(2) (b) and Zorbax SB-18 (c) columns.

whereas the Luna C18 and Zorbax SB-C18 columns were equally appropriate; nevertheless, the results obtained on the Zorbax SB-C18 column were superior to those the on the Luna C18 stationary phase as concerns its higher selectivity. Sample chromatograms measured on the three columns are presented in Figure 3.

Table I
Chromatographic Parameters of the Sample Peaks on the Three Columns

Column type	Parameter	Paracetamol	ASA	Papaverine
Hypersil ODS	k'	0.549	2.866	–
	α	0.000	5.224	–
	R	–	25.511	–
	t_R	2.323	5.799	–
Luna C18	k'	1.324	3.487	3.937
	α	0.000	2.634	1.129
	R	–	29.949	7.039
	t_R	3.486	6.731	7.406
Zorbax SB-C18	k'	0.662	2.932	4.102
	α	0.000	4.432	1.399
	R	–	30.844	16.192
	t_R	2.492	5.897	7.653

k' is the retention factor, α is the separation factor, R is the resolution and t_R is the retention time.

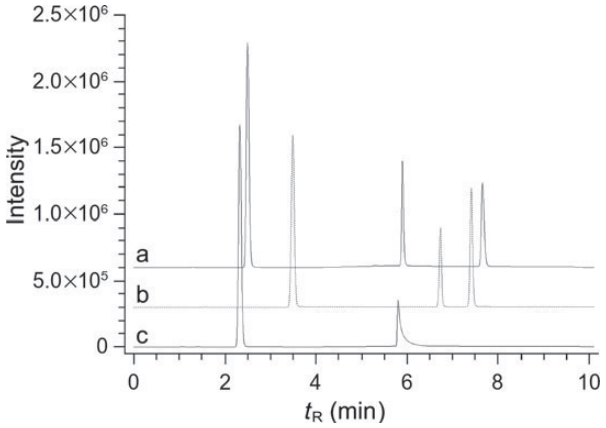

Figure 3. Comparison of the three stationary phases: (a) Zorbax SB-C18, (b) Luna C18 and (c) Hypersil ODS. It can be observed that papaverine was completely retained on the Hypersil ODS.

The developed method

The mobile phase during the quantitative determination was a potassium dihydrogenphosphate (25 mM, pH 3.43): ACN mixture. The details of the solvent gradient are to be seen in Table II. The buffer was prepared with potassium dihydrogenphosphate, and the pH of the solution was adjusted to the desired value with 1 M sulfuric acid solution. The flow rate was 1.5 mL/min, the run time was 10 min and the column temperature was 60°C. The chromatograms were recorded at 240 nm, at which wavelength all three components can be detected reproducibly. The choice of the detection wavelength was limited by the molar absorptivity of ASA, which is ~1 order of magnitude lower than those of the other components (29). Although ASA is the main component of the mixture, its peak intensity is lower than that of paracetamol. During runs, the UV spectra (200–300 nm) of the components were collected for identification of the drugs. The column applied during method validation was the Zorbax SB-C18 150 × 4.6 mm, 3.5 μm column.

Validation

We present a full validation of the method according to ICH guideline Q2 (R1) (30), including linearity, repeatability, intermediate precision, accuracy, specificity and robustness. As the

Table II
Solvent Gradient in the Chromatographic Method Described in Section Method Development Strategy

Time	ACN (%)
0.00	7
2.00	7
4.00	25
8.00	25
8.10	7
10.0	7

Table III
Results of the Accuracy Studies

Level		Paracetamol %			ASA (%)			Papaverine (%)		
		Rep.	Mean	RSD	Rep.	Mean	RSD	Rep.	Mean	RSD
70%	1.	103.0	102.5	0.45	100.8	100.1	0.61	98.4	98.1	0.26
	2.	102.1			99.8			97.9		
	3.	102.3			99.7			98.2		
100%	1.	101.4	101.9	0.43	98.3	98.5	0.17	98.2	98.7	0.47
	2.	102.3			98.7			98.9		
	3.	102.1			98.5			99.0		
130%	1.	100.7	101.7	0.94	97.0	97.6	0.59	95.8	96.8	0.90
	2.	102.5			98.1			97.4		
	3.	102.1			97.7			97.1		

method was to be utilized for the rapid quality control of dosage units, which does not require the method to be stability indicating, forced degradation studies were not conducted (31).

Linearity

The linearity of the method was examined in the concentration range between 0.02 and 0.04 mg/mL in the case of paracetamol, between 0.03 and 0.065 mg/mL for ASA and between 0.006 and 0.013 mg/mL for papaverine, these data corresponding to 70–130% of the nominal contents of the dosage units. The range was covered by the use of six solutions, each diluted from two individually prepared reference solutions, so that the sequence of the stock solutions used for the dilutions alternated. The peak areas determined with LCSolution were plotted versus the concentrations of the solutions and a straight line was fitted to the points. The slope of the paracetamol fitted straight line was 2.0171×10^8, the intercept was 1.5172×10^3 and R^2 was 0.9995. The slope of the fitted straight line in the case of ASA was 4.9169×10^7, the intercept was 4.9344×10^4 and R^2 was 0.9997. Finally, the slope of the fitted straight line for papaverine was 3.1811×10^8, the intercept was -3.6861×10^4 and R^2 was 0.9997. This demonstrated that in the studied concentration range the response of the method was linear.

Precision

Repeatability. Repeatability was checked on six individual samples according to the method described in Section Method development strategy. For paracetamol and ASA, RSD% proved to be 0.4 and 0.6%, respectively, both of which are acceptable. The papavarine results gave the highest RSD%, 1.4%, but this is also acceptable when the very low nominal amount of drug in the sample is taken into consideration.

Intermediate precision. The same analysis procedure was carried out by a different analyst on a different day, using a freshly prepared mobile phase.

The results for the paracetamol component were an RSD% of 0.7% and a relative difference of 1.3% between the averages of the repeatability (Day 1) and intermediate precision (Day 2) results compared with the mean of the average values measured for each. Both results can be accepted according to the principles of general pharmaceutical analytical practice. For ASA, the RSD% of the individual results was 0.9%, while the relative difference between the repeatability and intermediate precision was 1.2%. For papaverine, the RSD% proved to be 2.1% and the relative difference of the mean values on the 2 days was also 2.1%. All three results are in accordance with the appropriate guidelines, and are therefore considered acceptable.

Accuracy

The accuracy of the method was studied in the range between 70 and 130% of the nominal content of the powder. The results are shown in Table III. Although all of the average values fell between 95 and 105%, it should be mentioned that in the cases of ASA and papaverine most of the averages were $<100\%$, while in the case of paracetamol they were $>100\%$. This may raise a warning flag, but no trend was observed within the results that could be correlated with the increasing concentration of the sample groups.

Specificity

When the procedure was carried out with the solvent as blank (the sample contained the API without excipients), no peaks were detected at the retention times of the drugs.

Robustness

Examinations were made of the effects of changing the organic:aqueous ratio in the isocratic phases of the gradient, the pH of the aqueous phase, the flow rate of the mobile phase and the temperature of the column on the retention time and on the shapes of the drug peaks. The results of the robustness studies (Table IV) demonstrate that the ratio of the aqueous and organic phases exerted a great influence on both the retention time and the peak symmetry of the analyte. Variation of the pH of the aqueous phase caused only minor shifts in the retention times of the paracetamol and ASA peaks. The elution of paracetamol was not influenced by this parameter at all. In the cases of ASA and papaverine, the shift of the retention time in the opposite direction with the increase of pH caused an increase in resolution, which is in agreement with the increasing polarity of the components with pH. The flow rate change caused a minimal change in the retention time, proportional to the extent of the change. Flow rate changes did not influence the peak shape or plate numbers. Changes in column temperature did not cause significant changes in the retention times. Nevertheless, it is noteworthy that the retention of papaverine decreased with the decrease of temperature. Finally, variation of the organic:aqueous ratio, both at the start and at the end of the gradient, caused considerable changes in the peak retention times. Decrease of the organic modifier content of the initial hold increased the retention of paracetamol, while increase of the organic component pushed the peak very close to the void

Table IV
Results of the Method Robustness Tests

Condition changed		Paracetamol			ASA			Papaverine		
		t_R (min)	N	Symmetry factor	t_R (min)	N	Symmetry factor	t_R (min)	N	Symmetry factor
Column temperature (°C)	55	2.660	6380	1.261	6.324	69,377	1.445	7.122	50,747	1.597
	60	2.527	5540	1.237	6.213	69,445	1.460	7.160	53,197	1.588
	65	2.358	5600	1.264	6.032	81,057	1.423	6.992	60,963	1.542
Buffer pH	3.23	2.531	5843	1.249	6.308	68,186	1.476	6.933	53,433	1.629
	3.43	2.527	5540	1.237	6.213	69,445	1.460	7.160	53,197	1.588
	3.63	2.516	5690	1.245	6.072	75,013	1.412	7.252	52,516	1.563
Flow rate/(mL/min)	1.4	2.695	5521	1.242	6.453	68,816	1.437	7.459	51,720	1.604
	1.5	2.527	5540	1.237	6.213	69,445	1.460	7.160	53,197	1.588
	1.6	2.357	5346	1.265	6.000	70,952	1.449	6.900	53,720	1.595
Aqueous : organic ratio	5%	3.030	6408	1.210	6.324	77,103	1.418	7.132	54,830	1.583
	7%	2.527	5540	1.237	6.213	69,445	1.460	7.160	53,197	1.588
	9%	2.122	5865	1.319	5.986	69,115	1.476	6.947	54,415	1.567
	23%	2.516	6052	1.270	6.399	65,585	1.404	7.714	45,410	1.613
	25%	2.527	5540	1.237	6.213	69,445	1.460	7.160	53,197	1.588
	27%	2.502	6152	1.252	5.998	78,520	1.443	6.618	63,169	1.559

peak. Decrease of the organic modifier content at the end of the gradient increased the retention of both ASA and papaverine, this being more significant in the case of papaverine. On the other hand, the papaverine peak shape became more asymmetric and the number of theoretical plates also decreased in this case. A change in the opposite direction led to decreases in the retention times of ASA and papaverine, the greater effect being observed for papaverine and in this case the two peaks came too close to each other. This last change did not influence the retention of paracetamol; only a slight increase in the theoretical plate number was observed.

The results reveal that the method is robust, and the peaks are well separated and elute with acceptable symmetry within the studied boundaries of the parameters.

Discussion

The presented results clearly demonstrate that the most challenging part of the development was to find an appropriate stationary phase on which all three 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 the 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 methanol 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, we succeeded in finding a balance between retention and solubility by applying a 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 ~100 bar during the runs), which ensures a longer column lifetime. The method validation was carried out according to the current ICH guidelines. All the results satisfied the guideline requirements.

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IV.

Research Paper

Validated HPLC Determination of 4-Dimethylaminoantipyrine in Different Suppository Bases

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Suppositories are important tools for individual therapy, especially in paediatrics, and an instrumental assay method has become necessary for the quality control of dosage units. The aim of this work was to develop a rapid, effective high-performance liquid chromatography method to assay aminophenazone in extemporaneous suppositories prepared with two different suppository bases, *adeps solidus* and *massa macrogoli*. With a novel sample preparation method developed by the authors, 4-dimethylaminoantipyrine was determined in these suppository bases with 95-105% recovery. The measurements were carried out on a Shimadzu Prominence ultra high-performance liquid chromatography system equipped with a 20 µl sample loop. The separation was achieved on a Hypersil ODS column, with methanol, sodium acetate buffer (pH 5.5±0.05, 0.05 M, 60:40, v/v) as the mobile phase at a flow rate of 1.5 ml/min. The chromatograms were acquired at 253 nm. The chromatographic method was fully validated in accordance with current guidelines. The presented data demonstrate the successful development of a rapid, efficient and robust sample preparation and high-performance liquid chromatography method for the routine quality control of the dosage units of suppositories containing 4-dimethylaminoantipyrine.

Key words: HPLC, validation studies, 4-dimethylantipyrine, suppositories, analytic sample preparation

Suppositories are currently very popular formulations especially in paediatrics, where they can be used for the effective lowering of fever. The choice of a suppository as the mode of drug delivery is justified in all cases when oral delivery is impossible, that is an unconscious or vomiting patient, or in the case of infants. The therapeutic effect of correctly applied suppositories can be compared with that of injections because the active substance can penetrate from the lower tract of the rectum to the inferior vena cava, thereby avoiding the vena portae and the liver, and can exert a systemic effect when transported to the vena cava inferior. Currently suppositories are actuality used therapeutically all over the world. Suppository is being widely used for several indications utilising its feature that local effect of the suppository can be transformed into therapeutic benefit (e.g. in case of treatment of ulcerative colitis, ulcerative proctitis or colorectal cancer in paediatric practise)[1-3]. Treatment of acute malaria in case of children requires combination therapy in order to avoid development of multidrug resistance. In these scenarios it is a plausible solution to deliver one of the drugs of the combination in suppository[4,5]. Thus rapid systemic effect can be achieved. For the delivery of several nonsteroid antiinflammatory drugs, such as paracetamol or indomethacin, efficacy of suppository form is equivalent with or superior to oral route[6,7]. Therefore, the quality control of this widely applied dosage form with a modern, instrumental analytical method is highly desired.

In Hungarian pharmaceutical practice, extemporaneous products including suppositories are just as popular as factory-produced medicines. Extemporaneous products comprise part of personal therapy, and take into account the physical status, age and other diseases of the patient. Extemporaneously produced pharmaceuticals are used particularly in paediatric clinical departments, where they are the most effective means of lowering fever.

4-Dimethylaminoantipyrine (aminophenazone, AMFZ) is an antipyretic and analgesic that is

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frequently used in paediatric practice in Hungary. This molecule is also referenced in literature as 4-dimethylamino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-on, aminopyrin, pyramidon, dimethylaminophenyldimethylpyrazolon, 2,3-dimethyl-4-dimethylamino-1-phenyl-5-pyrazolon or dimethylaminophenazon.

Numerous manufacturers market antipyretic suppositories in various dosage strengths for neonates and infants. The antifebrile effect of AMFZ develops especially quickly (comparable to that of injections) if the drug is taken rectally. An additional benefit is that its administration does not require specially trained staff.

Agranulocytosis, one of the registered side-effects of the substance, has a very low incidence, while carcinogenicity, another possible side-effect, can be completely eliminated through rectal administration[8-16]. During its biotransformation[8-16], AMFZ is demethylated[13,16,17] in two steps, catalysed by cytochrome P450 2B[9,10]. The demethylated product then undergoes acetylation[12] and is eliminated from the body as acetylaminoantipyrine. In the presence of nitrite ion at pH between 2.0 and 3.1, the carcinogenic nitrosamine derivative dimethylnitrosamine is formed[8] in parallel with the demethylation. The physiological circumstances in the stomach provide a suitable medium for this reaction to take place[17-19]. In contrast, the rectal administration of AMFZ completely eliminates the possibility of dimethylnitrosamine formation as the pH of the mucous fluid in that region is around 7.9.

In consequence of the increased application of suppositories, there is a current demand for a rapid, effective and state-of-the-art reversed-phase chromatographic method for routine analysis. Our literature search revealed that methods for the high-performance liquid chromatography (HPLC) analysis of AMFZ are very rare and those found related to very low concentrations in biological fluids or tissues[20-23]. Our aim was therefore to develop and validate a suitable general sample preparation and chromatographic method for suppositories containing AMFZ. Moreover, HPLC analysis of the pyrazolone derivative metamizole in tablet formulations could shed light on the initial steps of method development[23-27].

MATERIALS AND METHODS

Throughout this investigation, HPLC grade organic solvents were used. Methanol was obtained from Merck (LiChrosolv, Darmstadt, Germany) and Sigma-Aldrich (Chromasolv for HPLC, St. Louis, MO, USA). The buffer solutions were prepared by using triple distilled water. The pH of the buffer solutions was set to the desired value by using sodium acetate anhydrate (Reanal) and acetic acid 96% (Molar Chemicals). 4-Dimethylaminoantipyrine (Sigma-Aldrich) was used as reference substance.

Suppositories based on *adeps solidus* (hard fat; Ph. Eur. 7.4) or on *massa macrogoli* (a hydrophilic suppository base sorbitan monolaurate and macrogol 1540 in 95:5 w/w ratio), without or with the active substance (100 mg of AMFZ), were utilised for the accuracy and specificity studies of the validation and during the method development; they were provided by the Pharmacy of the University of Szeged.

Repeatability and intermediate precision studies were carried out with *Suppositorium antipyreticum pro parvulo* prepared according to *Formulae Normales* VII. (a collection of standard prescriptions in Hungary), produced by Naturland Hungary Ltd. (Budapest, Hungary), in order to ensure the best homogeneity of the samples.

Paediatric suppositories that contain 100 mg of AMFZ exclusively are not marketed by pharmaceutical companies. These reference suppositories for the repeatability and intermediate precision measurements were therefore provided by the Institute of Pharmaceutical Technology of the Faculty of Pharmacy, University of Szeged, prepared as prescribed by the authors: 12 suppositories were prepared containing 100 mg of AMFZ in each dosage unit. All dosage units used during the method development and validation were prepared through the use of moulds for infant suppositories.

Instruments and conditions:

All measurements were carried out using a Shimadzu Prominence (Shimadzu Corp., Japan) ultra high-performance liquid chromatography (UHPLC) system, consisting of an LC-20AD high-pressure pump equipped with a 4-way solenoid mixing valve, a CT0-20A column thermostat and an SPD-M20A UV/Vis Diode Array detector equipped with a 10

mm optical path length flow cell. The injections were carried out via a Rheodyne manual injection valve fitted with a 20 µl sample loop. The separation was achieved on a Hypersil ODS (C18) 150×4.6 mm, 5 µm column (Thermo Scientific, Keystone, UK). The flow rate of the mobile phase was 1.5 ml/min. The composition of the mobile phase was selected on the basis of the results obtained from Pallas chromatographic prediction software[21]. The mobile phase was methanol–sodium acetate buffer (pH 5.5; 0.05 M) (60:40, v/v). The chromatograms were acquired at 253 nm for 5 min. The chromatograms were integrated by means of LCSolution software (Shimadzu Corp.)

Chromatographic method development:

The stationary phase was chosen on the basis of the work of El-Seikh *et al.*[20], but our initial experiments revealed that the composition and the pH of the mobile phase had to be changed considerably. With methanol–acetic acid (pH 2.78; 1.0%) (70:30, v/v) as mobile phase, the AMFZ peak eluted between 15 and 30 min and showed significant asymmetry. It was obvious that the mobile phase composition described by El-Seikh *et al.* would have given a much longer retention time. Simulations carried out with the Pallas software[28] showed that the pH should be >4.5 to achieve acceptable robustness and peak shape. A set of experiments was therefore designed using methanol–sodium acetate buffer (pH 4.5 or 5.0; 0.05 M, 50:50 or 60:40, v/v) as mobile phase in the various combinations. The shape of the AMFZ peak in the resulting chromatograms improved on increase of both the pH and the proportion of methanol. In the final experiment, with methanol–sodium acetate buffer (pH 5.5; 0.05 M) (60:40, v/v) as eluent, the symmetry factor of the AMFZ peak proved to be 1.43, and the peak width measured at the baseline was 0.2 min. It still seemed plausible to use acetate buffer at pH 5.5, where it has a somewhat lower buffer capacity, but the chosen concentration of 0.05 M compensates this.

Preparation of samples for analysis:

Extemporaneous prescriptions do not usually specify the suppository base to be used as vehicle and it is left to the pharmacist to apply his or her professional knowledge to choose the most suitable one from the possibilities listed in the official Pharmacopoeia. The development of the sample preparation involved in particular two suppository vehicles, *adeps solidus* and *massa macrogoli*, as these are the most commonly chosen ones. The same methanol–water solvent mixture (50:50, v/v) was used for both vehicles. However, the methods differed as concerns other aspects of the sample preparation. This is due to the fundamentally different physico-chemical properties of these two vehicles.

Adeps solidus and *massa macrogoli* cannot be distinguished by purely organoleptic examination. In the first step of sample preparation, the suppository (containing the unidentified vehicle) was weighed in a beaker, 15 ml of the above solvent mixture was added, and the beaker was heated in a 40° water bath until the suppository melted. (At this point, the behaviour of the molten suppository reveals its nature). In the case of *adeps solidus*, a consistent, clear, colourless fatty phase appears on the surface of the solvent mixture, whereas with *massa macrogoli* the solution becomes homogeneous and clear and no second phase can be observed. In some cases, *massa macrogoli* may contain a certain amount of tensides, when the resulting solution is opaque, but even then no second phase or fat droplets can be observed.

At this stage, the active substance was extracted from the vehicle by shaking the sample for 10 min.

The *massa macrogoli*-based samples did not require filtration, so the solution was transferred directly into a 50 ml volumetric flask and the beaker was washed out with another 15 ml and then 2×5 ml of solvent mixture, the washings likewise being transferred to the volumetric flask, the solution next being made up to volume with the solvent mixture.

The *adeps solidus*-based samples required removal of the fatty phase by freezing on an ice-bath, when the fat solidified and the liquid could be decanted into a 50 ml volumetric flask. This extraction step was repeated with a second 15 ml portion of solvent mixture in a 40° water bath. The beaker was finally washed twice with 5 ml of solvent mixture, which was transferred to the volumetric flask, the solution then being made up to volume with the solvent mixture. The outstanding benefit of this sample preparation procedure is that it does not require an initial knowledge of the suppository base used.

Finally, in both cases a 0.3 ml aliquot of the stock solution was transferred to a 10 ml volumetric flask and made up to volume with the solvent mixture. The

solution was filtered on a Millipore Millex PVDF membrane filter with a pore size of 0.45 μm.

Preparation of standard solution and establishment of system suitability:
The AMFZ contents of the samples were quantified by reference to a standard AMFZ solution with a concentration of 0.075 mg/ml in the same solvent mixture, which corresponded to the theoretical 100% concentration level of the sample solutions to be examined. Two standard solutions were prepared from independent stock solutions in order to check the system suitability by the following procedure.

The precision of the injections was checked before all measurement sets by injecting the first standard solution five times. The system was considered suitable if the relative standard deviation percent (RSD%) of the five replicate injections did not exceed 2.0%. The accuracy of the calibration was checked by injecting the second standard solution twice. The results were accepted if the correlation factor calculated from the average response ratio of the two standard solutions did not exceed 2.0.

Correlation factor was calculated by the following formula, [1-(AStd1×wStd2)/(AStd2×wStd1)]×100%; where AStd1 and AStd2 are the average peak areas of the replicate standard injections, while wStd1 and wStd2 are the weights of the standard substances used to prepare the solutions. The symmetry factor of the main peak of interest was also monitored throughout the measurements; it had to be between 0.7 and 2.0 for the analysis to be started.

Validation:
A full validation of the method according to International Conference on Harmonisation (ICH) guideline Q2 (R1)[29], including linearity, repeatability, intermediate precision, accuracy, specificity and robustness have been performed. As the method was to be utilised for the rapid quality control of dosage units, which does not require the method to be stability-indicating, forced degradation studies were not conducted[30]. The repeatability, intermediate precision, accuracy and specificity studies were carried out with both vehicles.

Linearity:
The linearity of the method was examined in the concentration range between 0.025 and 0.150 mg/ml, which corresponds to 50–450% of the nominal content of the suppositories. The higher limit was chosen with regard to the fact that initial experiments gave individual results in this concentration range. Thus, it was necessary to check the method at extremely high active substance concentrations. The range was covered by seven solutions each diluted from two individually prepared reference solutions so that the sequence of the stock solutions used for the dilutions alternated. The peak areas determined with LCSolution were plotted versus the concentration of the solutions and a straight line was fitted to the points. The slope of the fitted straight line was found to be 3.498 107, the intercept was -5.165 104 and R^2 was 0.9998. This proved that in the proposed concentration range the method was linear.

Precision and repeatability:
Repeatability was checked on six individual suppositories prepared according to the method described in chromatographic method development section. In the case of *adeps solidus* as vehicle, one of the six replicate results exceeded the 125% limit, and this result was omitted from the calculation of the RSD%. On the basis of our result that there is no carryover between the injections and the fact that active substance was not added to the solution. RSD% proved to be 1.4%, which can be considered acceptable when it is taken into account that each sample preparation was made from different individual suppositories and not from a composite sample of multiple suppositories. The *massa macrogoli*-based suppositories gave an RSD% of 2.1%, which is also acceptable.

Intermediate precision:
The same analytical procedure was carried out by another analyst on another day, using a freshly prepared mobile phase. Relative differences between average results of 2 days were calculated with the following formula: ($\bar{X}$Day1-$\bar{X}$Day2)×2/($\bar{X}$Day1-$\bar{X}$Day2)×100%, where $\bar{X}$Day1 denotes the average result of the specific day. The results for the *adeps solidus*-based samples were an RSD% of 1.2% and a relative difference of 1.3% between the averages of the repeatability (Day 1) and intermediate precision (Day 2) results compared to the mean of the average values measured for each. Both results can be accepted according to the principles of general pharmaceutical analytical practice. For the *massa macrogoli*-based samples, the RSD% of

the individual results was 2.5%, while the relative difference between the repeatability and intermediate precision was 3.7%. Both results are in accordance with the appropriate guidelines, and are therefore considered acceptable.

Accuracy:

The accuracy of the method was studied between 50% and 450% of the nominal content of the suppositories, that is, 100 mg. The results are shown in Table 1. Although all of the average values fell between 95% and 105%, it should be mentioned that in the case of *adeps solidus* most of the averages were below 100%, while in the case of *massa macrogoli* they were above 100%. This may raise a warning flag, but there was no trend within the results that could be correlated with the increasing concentration of the sample groups.

Stability of standard and sample solutions:

Stability of the standard solution and the sample solution was studied for 4 days. Both solutions were stored in a refrigerator between 2° and 8°. The acceptance criterion was set up according to the relative difference value defined by the following formula: [(AStart-AStored)/AStart]×100%. The solution was considered stable as long as the relative difference at a specific time point was lower than 3.0%. On the basis of the data presented in Table 2, the standard solutions can be considered stable for at least 96 h, and the sample solutions can be considered stable for at least 96 h.

Specificity:

When the procedure was carried out with blank suppositories (containing no active substance), no peak was detected at the retention time of AMFZ. It can be stated that there are so excipients in either vehicles that interfere with the determination of AMFZ.

Robustness:

The effects of changing the organic–aqueous ratio, the pH of the aqueous phase, the flow rate of the mobile phase and the temperature of the column on the retention time and on the shape of the AMFZ peak were examined. The results of the robustness study presented in Table 3 demonstrate that the ratio of the aqueous and organic phases exerted a great influence on both the retention time and the peak symmetry of the analyte. The pH of the aqueous phase significantly changed the symmetry of the peak, which is in accordance with the results obtained from the simulations with the Pallas software. The lower the pH, the more asymmetrical the peak was. In contrast, the pH of the mobile phase had only a very

TABLE 1: RESULTS OF THE ACCURACY STUDIES

Level %	Adeps solidus				Massa macrogoli			
	Replicates %		Mean %	RSD%	Replicates %		Mean %	RSD%
50	1.	98.7	99.4	0.63	1.	104.10	102.5	1.56
	2.	99.9			2.	100.90		
	3.	99.6			3.	102.40		
100	1.	99.5	100.3	0.75	1.	97.40	99.7	1.99
	2.	100.3			2.	100.50		
	3.	101.0			3.	101.10		
150	1.	96.0	95.3	1.36	1.	104.60	104.9	0.42
	2.	93.8			2.	105.40		
	3.	96.1			3.	104.70		
300	1.	96.2	95.5	0.87	1.	104.60	102.7	2.43
	2.	95.8			2.	103.70		
	3.	94.6			3.	99.90		
450	1.	96.3	96.5	0.55	1.	102.80	102.5	1.49
	2.	96.1			2.	103.80		
	3.	97.1			3.	100.80		

TABLE 2: RESULTS OF THE SOLUTION STABILITY STUDIES

Time (h)	Standard solution		Sample solution	
	Area	Relative difference (%)	Area	Relative difference (%)
0	2 903 843	-	2 076 275	-
18	2 909 154	0.2	2 075 720	0.0
24	2 904 895	0.0	2 073 721	-0.1
39	2 902 092	-0.1	2 074 466	-0.1
48	2 904 450	0.0	2 073 840	-0.1
63	2 906 272	0.1	2 074 709	-0.1
72	2 903 753	0.0	2 080 587	0.2
96	2 904 386	0.0	2 077 305	0.0

TABLE 3: RESULTS OF THE ROBUSTNESS STUDIES

Condition changed (Units)	t_R (min)	*N*	Symmetry factor
Aqueous: Organic ratio			
45:55	2.088	3973	1.535
40:60	1.761	4074	1.434
35:65	1.616	4512	1.460
Buffer pH			
5.00±0.05	1.837	3747	1.602
5.50±0.05	1.761	4074	1.434
6.00±0.05	1.846	4441	1.346
Flow rate (ml/min)			
1.3	2.021	4346	1.432
1.5	1.761	4047	1.434
1.7	1.676	4117	1.405
Column temperature (°C)			
25	1.785	3890	1.433
30	1.761	4074	1.434
35	1.751	4340	1.408

slight effect on the retention time of the peak. The flow rate influenced the retention time, as expected, while it had a negligible effect on the peak shape. The column temperature did not influence either the retention time or the symmetry of the main peak. The acquired chromatograms can be seen in fig. 1.

RESULTS AND DISCUSSION

On the basis of the results presented here the proposed method is appropriate for the determination of AMFZ in the concentration range 0.025-0.150 mg/ml with excellent repeatability, intermediate precision and accuracy. The chromatographic method is robust with respect to changing the parameters between the boundaries presented in Table 3. Retention parameter changes of the AMFZ peak are in excellent agreement with the expected behaviour in case of changing the flow rate, column temperature or ratio of the organic modifier. Changing the buffer pH did not influence the retention time or number of theoretical plates of AMFZ but had significant effect on the peak shape of the component.

The data presented in this paper reveal 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.

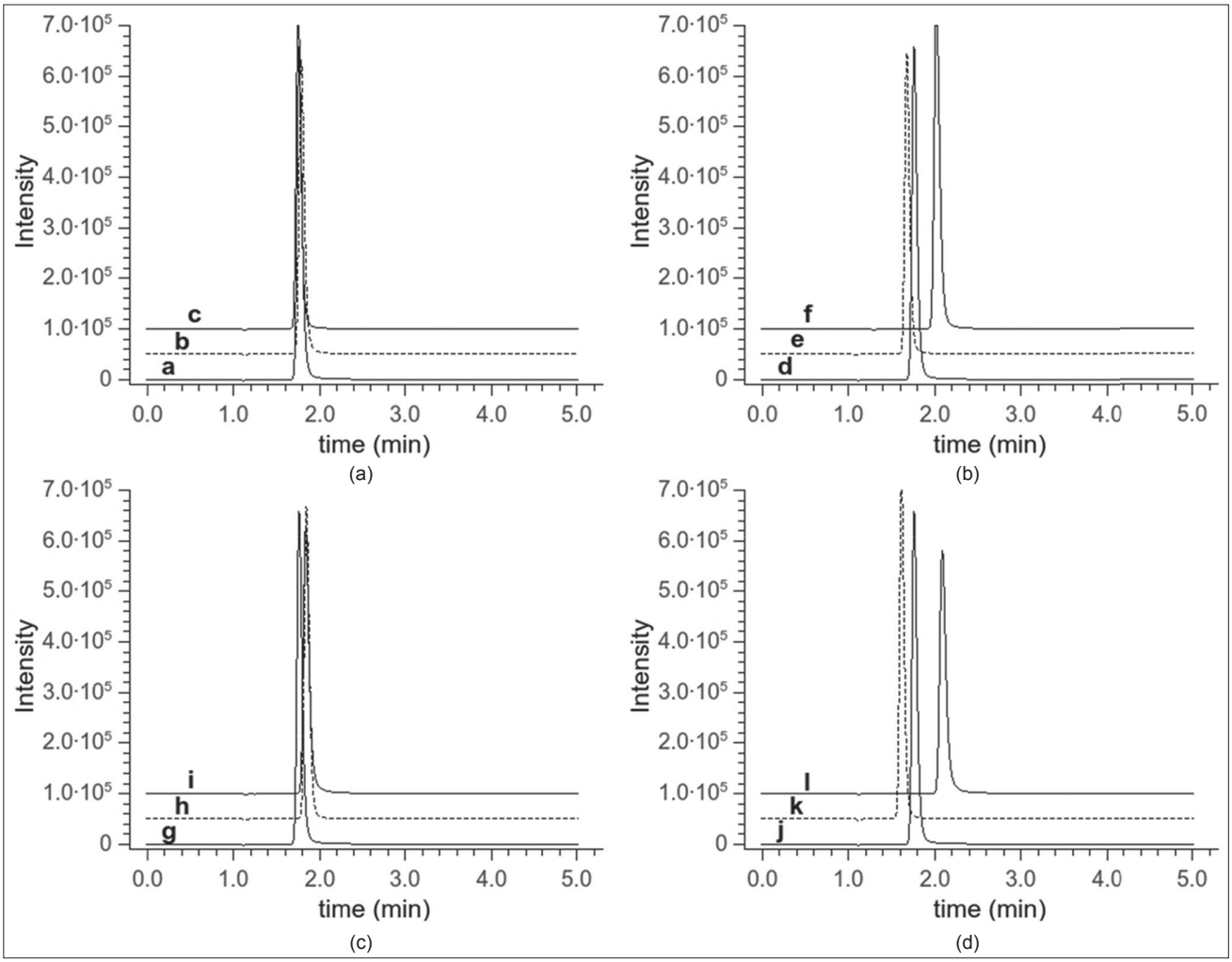

Fig. 1: Chromatograms of robustness test
Curves (a, d, g, j) show the initial condition. Curves (b, c) show the effect of column temperature variation (25°, 35°, respectively). Curves (e, f) show the effect of flow rate change (1.3 ml/min, 1.7 ml/min, respectively). Curves (h, i) show the effect of mobile phase pH (5.0, 6.0, respectively). Curves (k, l) show the influence of organic modifier percent (55%, 65%, respectively).

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