Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 53–60
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of a high-performance-liquid-chromatographic method for the determination of biapigenin in biorelevant media

S. Schulte-Löbbert, K. Westerhoff, A. Wilke, M. Schubert-Zsilavecz, M. Wurglics *

Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University Frankfurt, Marie-Curie-Straße 9, 60439 Frankfurt/Main, Germany

Received 6 December 2002; received in revised form 25 March 2003; accepted 30 March 2003

Abstract

A new precise, rapid and selective high-performance-liquid-chromatographic (HPLC) method has been developed to quantify biapigenin in St. John's Wort (SJW) preparations and to investigate its release characteristics in the dissolution test using both compendial and biorelevant media. Experiments were carried out on a LiChroCart® 125-4, RP-18 (5 μm) column, using gradient elution at a flow rate of 1 ml/min. The binary mobile phase consisted of solvent A (acetic acid, 5:100, w:w) and B (a mixture of acetonitrile and methanol (3:1, v:v)). Detection was performed at a wavelength of 270 nm using a photodiodearray detector. The limit of detection was 0.05 μg/ml, the injection volume 20 μl. Five SJW preparations were chosen to determine the amount of biapigenin in the dosage form and to investigate their release characteristics. Best results in terms of release as well as discriminating the tested products were obtained, using fed state simulated intestinal fluid (FeSSIF), where over 80% of biapigenin was dissolved after 20 min comparing to 70% using simulated gastric fluid sine pepsin (SGF_{sp}) as compendial medium. Experiments in fasted state simulated intestinal fluid (FaSSIF) show 80% release of biapigenin within 80 min.

Keywords: *Hypericum perforatum*; St. John's Wort; Biapigenin; Dissolution; FeSSIF; FaSSIF; SGF_{sp}

1. Introduction

Hypericum perforatum (St. John's Wort (SJW)) counted among the favorite herbal drugs used for the treatment of mild to moderate depression. Several clinical studies have been conducted to verify effectiveness of the extract [1]. The major active constituents of *H. perforatum* are considered to be hyperforin (a phloroglucinolderivate, 2–4%), hypericines (naphthodianthrones, 0.1–0.3%) along with a broad spectrum of flavonoids (2–4%), e.g. the quercetinglycosides rutin, hyperoside, quercitrin and isoquercitrin and its aglycone

* Corresponding author. Tel.: +49-69-798-29432; fax: +49-69-798-29352.
E-mail address: wurglics@pharmchem.uni-frankfurt.de (M. Wurglics).

0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00263-2

quercetin, as well as biflavonoids (0.1–0.5%). The biflavonoids in *H. perforatum*, seldom found in nature, are represented by biapigenin (Fig. 1) and amentoflavone [2]. Biapigenin, first isolated in 1986 [3] is mainly located in the buds and flowers of *H. perforatum*. There is a progressive increase of biapigenin content in developing blossom buds reaching its maximum in the phase just before blooming. After the flowers have opened the biapigenin content continuously decreased. This may be attributed to the finding that biapigenin is mainly located in the pollen, which are continuously lost [4].

Furthermore several studies have been carried out regarding the pharmacological behavior of biapigenin. Radioligand binding techniques were used to examine the effect of SJW extract and constituents of SJW at various CNS receptors in vitro. It was shown that biapigenin inhibited ^{3}H-estradiol binding to the estrogen-α receptor with an IC_{50} value of 1 μM [5]. In a radio ligand binding assay biapigenin was able to inhibit norepinephrine and L-glutamate synaptosomal uptake with IC_{50} values >10 μM, serotonin reuptake with an IC_{50} value >100 μM, respectively [6]. In a drug interaction study it could be demonstrated that biapigenin is a potent, competitive inhibitor of CYP3A4, CYP2C9 and CYP1A2 with K_i values of 0.038, 0.32 and 0.95 μM, respectively [7]. In in vitro binding studies biapigenin inhibited ligand binding at benzodiazepine receptors with an IC_{50} of 2 μM [8]. Moreover biapigenin was remarkably active in the forced swim test (FST), an established screening model for antidepressants [9].

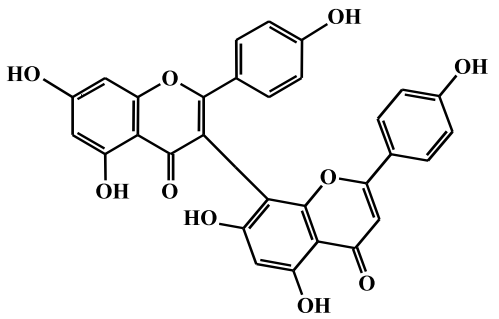

Fig. 1. Structure of biapigenin.

Recent studies indicate that the components of SJW extract may synergistically combine to produce the antidepressive effect. Thus, neither rutin alone nor SJW extracts with a reduced rutin content are therapeutically effective, in contrast to SJW extracts containing usual levels of rutin [10].

To check the quality of pharmaceutical products it is necessary to determine the content of the pharmaceutical active ingredients and then to evaluate the dissolution profile of the whole spectrum of substances. In previous studies the dissolution profiles of several SJW products regarding release of hyperforin and flavonoids were published [11].

The aim of this study was to establish an HPLC method to quantify biapigenin in SJW extracts and to investigate its release characteristics from selected SJW products currently available on the German market, using both compendial and biorelevant media. Although several HPLC methods [12,13] and one HPLC-MS method [14] already exist to quantify biapigenin, they were not suited to cope with the large number of samples which need to be measured during the dissolution test to characterize the dissolution profile.

Therefore, a new rapid HPLC method was established, which allowed the determination of biapigenin within only 17 min.

2. Experimental

2.1. Instrumentation

HPLC was performed on a Varian ProStar system with two solvent delivery modules (model 210), a PDA detector (model 330), a Varian ProStar autosampler (model 410) and a Degasys DG-1210 degasser. All system operations as well as data acquisition and integration were controlled with a Varian Star Chromatography Workstation. The analyses were carried out on a LiChroCart® 125-4, RP-18 (5 μm) column, using a gradient elution. Different mobile phases were tested in order to find the best conditions for separating biapigenin from the other extract constituents. The

optimal composition of the mobile phase was determined to be: solvent A, acetic acid (5:100, w:w) in Milli-Q water; solvent B, a mixture of acetonitrile and methanol (3:1, v:v). During the first 7 min the composition of the mobile phase changed from 30% solvent B to 100% solvent B. This composition was kept constant for the next 3 min, then changed to 30% B again. The column was reequilibrated for 7 min until the next sample was injected. All solvents were degassed with helium before they were used for analysis. The flow rate of 1 ml/min resulted in a back pressure of approximately 90 bar. UV detection was carried out at 270 nm, the absorption maximum of biapigenin, and the injection volume was 20 μl.

2.2. Solvents and chemicals

Pure biapigenin (purity 93.5%), used as the reference standard was provided by Dr Wilmar Schwabe Arzneimittel GmbH (Karlsruhe, Germany). SJW products (listed in Table 1) were purchased from a pharmacy in Frankfurt am Main, Germany. Acetonitrile, ethanol and methanol were HPLC gradient grade and supplied by Carl Roth (Karlsruhe, Germany). Water was purified by a Milli-Q system (Millipore, Bedford, USA) and used for all aqueous procedures. All other chemicals used for the dissolution test were analytical grade.

2.3. Standard stock solutions and dilutions

Standard stock solutions of 300 μg/ml biapigenin was prepared by dissolving biapigenin reference standard in methanol. Three aliquots of the stock solution were taken and diluted with the eluent (acetonitrile–methanol (60:40, v:v)) to obtain three sets of dilutions, containing nine different concentrations in the range 0.01–30 μg/ml. The stock solutions were stored in a deep freezer at −20 °C.

2.4. Quantification

Calibration was based on external standardization, with the peak area as assay parameter. Standard calibration curves were constructed by plotting the corresponding peak area against six standard concentrations of biapigenin in media and in eluent, respectively. All statistical data were calculated using MICROSOFT EXCEL 97.

2.5. Sample preparation

From each tested product 10 U from a single batch were weighed and milled together in an analytical mill. Regarding the capsule preparation the content of the capsule was emptied and mixed in an analytical mill. A tenth of the total mass was then quantitatively transferred into a 50 ml volumetric flask containing 40 ml ethanol (80:100, v:v). Extraction of the analytes was completed by subjecting the flask to ultrasound at 30 °C for 10 min. The volume in the volumetric flask was brought up to 50 ml with ethanol (80:100, v:v). Samples of the resulting supernatant solutions were removed by syringe and filtered through 45 μm filters (Rezist 30/0.45; Schleicher & Schuell, Germany). An aliquot (200 μl) of the filtered sample solution was transferred into an HPLC vial, containing 300 μl ethanol (80:100, v:v) and

Table 1
SJW preparations

Product	Dosage form	Extraction solvent	DER[a]	Extract/dosage form (mg)	Biapigenin/dosage form (mg)	Biapigenin/extract (%)
Texx® 300	Film tablet	MeOH 80%	4–7:1	300	0.49	0.16
Neuroplant® 300	Film tablet	EtOH 60%	2.5–5:1	300	0.73	0.24
Felis® 425	Capsule	EtOH 60%	3.5–6:1	425	1.29	0.3
Laif® 900	Film tablet	EtOH 80%	3–6:1	900	3.81	0.42
Felis® 650	Film tablet	EtOH 60%	3.5–6:1	650	1.48	0.22

[a] Drug:extract ratio.

500 μl of mobile phase (acetonitrile–methanol, (60:40, v:v)).

2.6. Dissolution

The USP Apparatus 2 (Paddle) was used for all dissolution studies. Release in 500 ml of medium at a temperature of 37 °C and a stirring rate of 100 rpm was investigated. Five-ml samples were removed at predetermined times with a Fortune Optima® syringe. The samples were then filtered with 0.45 μm filters and analyzed by HPLC.

2.7. Media

Simulated Gastric Fluid (USP XXIII) sine pepsin (SGF_{sp}) was used as the compendial medium. The pH of this medium is 1.2. In addition the dissolution in two simulated intestinal media, fasted state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) was also studied. These two biorelevant media were developed to simulate the conditions in the intestine in the fasted and fed state, respectively [15], and contain appropriate concentrations of bile components as well as having higher pH values than SGF_{sp}. The pH value of FaSSIF is 6.5, FeSSIF 5.0, respectively.

2.7.1. Calibration procedure

Validation of the HPLC method was conducted according to ICH/Q 2A [16].

2.7.2. Specificity

To determine the analyte unequivocally in the presence of other components which may be present (impurities, degradants, media) chromatograms of diluent (ethanol (80:100, v:v)), eluent (acetonotrile–methanol (60:40, v:v)), standard solution (10 μg/ml), a placebo solution (mixture of adjuvants), sample solution and different dissolution samples (using FeSSIF, FaSSIF and SGF_{sp}) were compared. The HPLC chromatogram of SJW extract in Fig. 2A indicates the complete separation of biapigenin from other components of SJW extract. The retention time was approximately 6.3 min. Fig. 2B shows a typical chromatogram obtained after spiking FeSSIF with biapigenin (same results when spiking FaSSIF and SGF_{sp}, Figures not shown). The chromatogram obtained from the dissolution test (Fig. 2C) demonstrates the good separation of biapigenin from all ingredients and excipients of a SJW product. To confirm the specifity of the methode the UV spectrum of biapigenin standard was compared with the UV spectrum obtained from the peak eluting at 6.3 min. The UV spectra were the same, having the absorption maximum at 270 nm. The spectra at the maximum, ascending and descending part of the peaks were identical and a further indication of peak purity. There was no interference detected when comparing the chromatograms of blank and spiked media samples.

2.7.3. Precision

To ascertain the precision of the developed method the intraday, interday and intermediate method repeatability was tested. To check the intraday precision six different samples from ten milled dosage forms were prepared within the same day. The relative standard deviation (R.S.D.) was 2%. For evaluating the interday precision six different samples of ten milled dosage forms were prepared on separate days. The value for the interday precision of the method given by R.S.D. was 1.68%. Finally a different laboratory worker prepared six samples of ten milled dosage forms to test the intermediate precision of the method. The result given by R.S.D. was 1.45%. These values were within the limits recommended for analytical method validation.

2.7.4. Linearity

Calibration curves were done in eluent and in each dissolution medium. The concentration range of the calibration curve in eluent was 3–30 μg/ml and was used for determination of the biapigenin content in the SJW preparations. The calibration curves in the media were in the range 0.3–3 and 3–30 μg/ml. Depending on how much biapigenin was dissolved during dissolution in the tested medium either the low or the high calibration curve was used for quantification of biapigenin. All calibration curves were done with six concentrations of biapigenin.

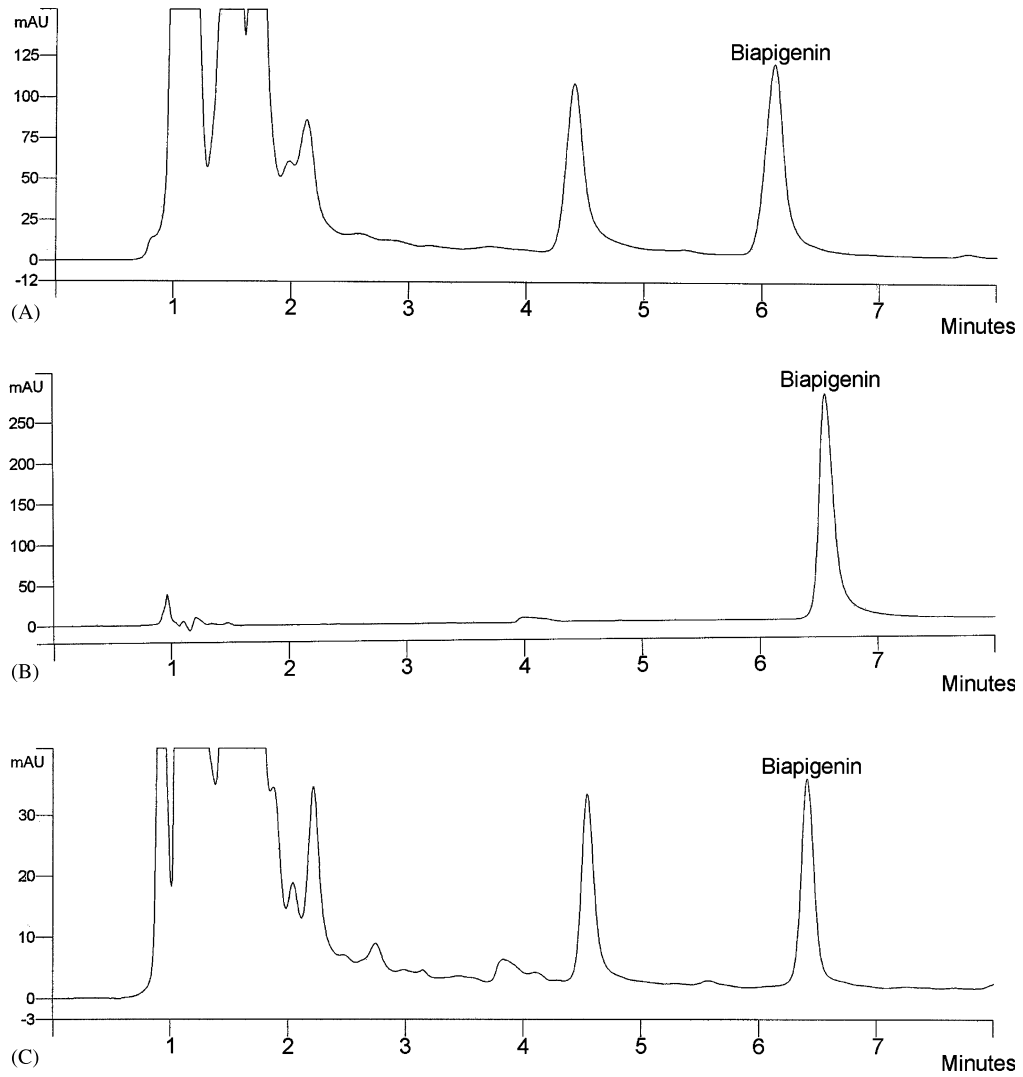

Fig. 2. Chromatograms of biapigenin in various media. (A) Ethanolic sample of SJW product containing biapigenin. (B) FeSSIF sample spiked with biapigenin standard stock solution (30 μg/ml). (C) Dissolution sample of SJW product using FeSSIF.

2.7.5. Detection and quantification limits

Based on a signal-to-noise ratio of 3:1 the limit of detection (LOD) was 0.05 μg/ml, limit of quantitation (LOQ), based on a signal-to-noise ratio of 10:1, was 0.1 μg/ml for biapigenin dissolved in eluent. The LOQ in the media was 0.3 μg/ml.

2.7.6. Recovery

To confirm the accuracy of the proposed methode, recovery experiments were carried out by standard addition technique. Therefore, a known amount of standard was added to pre-analyzed ethanolic samples as well as dissolution samples in all tested media. The recovery of biapigenin was determined in triplicate and found to be 99.7–107.9% depending on the medium.

2.7.7. Stability

Stability tests were conducted at different storage conditions. Standard stock solution and extraction samples were stored in the deep freezer

at −20 °C as well as in the refrigerator at 4 °C for 40 days. The stability of biapigenin in extraction samples was studied at room temperature after 6 days and in medium after 24 h storage in the autosampler. Biapigenin was stable in all experiments.

2.7.8. Solubility

Solubility of biapigenin was determined in SGF_{sp}, FaSSIF and FeSSIF. Therefore, three sets of 200 mg SJW extract in 10 ml of each media were prepared and stored at 37 °C in a shaking water bath. Samples were removed after 12 and 24 h. Best results in solubility were obtained after 24 h using simulated intestinal fluid. While 14.91 μg/ml biapigenin was dissolved in FeSSIF and 9.97 μg/ml biapigenin was determined in FaSSIF after 24 h, only 2.49 μg/ml biapigenin could be quantified in SGF_{sp}.

3. Results and discussion

After completing validation, the assay was tested for its analytical suitability by evaluating the dissolution profile of biapigenin in SJW preparations. Five SJW products were chosen to determine the amount of biapigenin in the tablets/capsules and to investigate their release characteristic in SGF_{sp}, FaSSIF and FeSSIF. The amount of biapigenin in the tested products ranges between 0.16 and 0.42% in the extract. Dissolution results are expressed in terms of percent release to the content of the preparations measured previously. As Fig. 3 shows best results were obtained using FeSSIF, representing the fed state conditions in the small intestine. 80% of biapigenin was released after 20 min of Laif® 900, compared with 70% using SGF_{sp}. The reason that the dissolution profile of Laif® 900 using SGF_{sp} has its maximum at 70% release is due to the fact that biapigenin is less soluble in SGF_{sp} than in FaSSIF and FeSSIF. The dissolution profile of the products using FaSSIF is not as uniform as using FeSSIF or SGF_{sp}. However, biapigenin was released up to approximately 90% in FaSSIF after 90 min.

As mentioned before previous studies were conducted to evaluate the dissolution behavior of the other pharmaceutical relevant components of SJW extract [17]. The results of this study show, that one of the active components, hyperforin, is only released adequately under fed state conditions. Therefore, the dissolution behavior of all further SJW products were conducted in FeSSIF. The results are presented in Fig. 4. After 120 min 100% biapigenin of Laif® 900, 90% of Felis® 650, 70% of Neuroplant® 300, 65% of Texx® 300 and 60% biapigenin of Felis® 425 was dissolved.

Regarding the FIP recommendation on biopharmaceutical characterization of herbal medicinal products (HMPs), plant extracts can be classified according to the knowledge available on their composition and efficacy into three categories (A, B1 and B2) [18]. SJW products depending on the pharmaceutical–analytical, pharmacological–toxicological, and clinical findings can be identified as type B1 herbal drug preparations. These extracts contain chemically defined constituents, which possess relevant pharmacological properties and are likely to contribute to the clinical efficacy. However, evidence that they are solely responsible for the clinical efficacy is not yet available. Quality of HMPs is determined by the raw material as well as the manufacturing process leading to finished products which guarantee batch to batch reproducibility regarding their pharmaceutical active ingredients. In previous studies it could be demonstrated that some SJW products on the German market fulfill this requirement, other products on the other hand exhibit wide batch to batch variability in hyperforin and hypericin content [19]. In vitro dissolution testing of HMPs is performed to determine the releasing rate of drug substances in predetermined time. Although the European Agency for the Evaluation of Medicinal Products (EMEA) note for guidance [20] does not define explicitly HMPs containing extracts with constituents of known therapeutic activity (category A) or pharmacologically relevant constituents (category B1) dissolution tests for these categories are feasible. Even though dissolution tests for SJW products are not explicitly required by law as a feature to

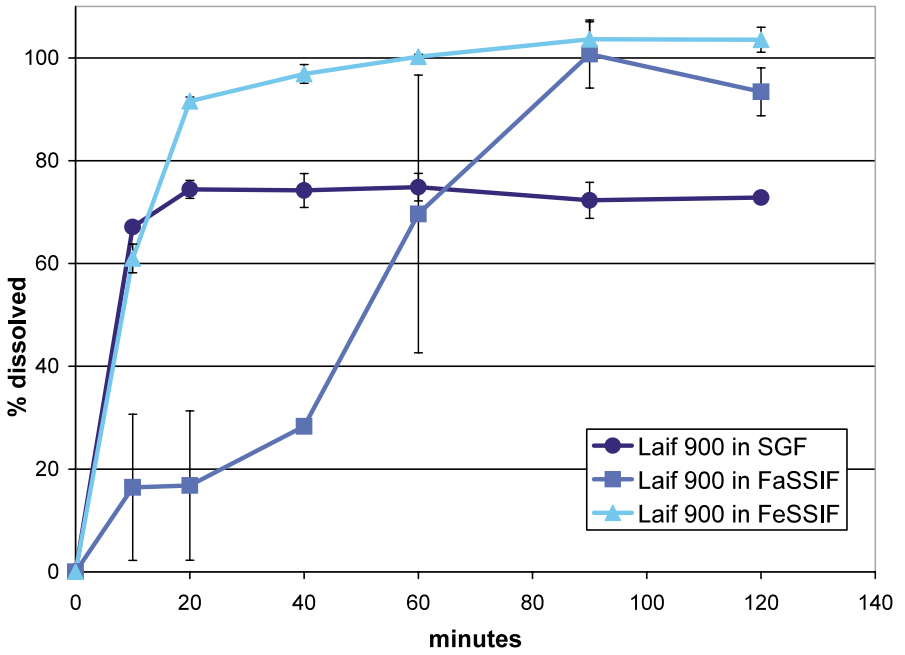

Fig. 3. Release of biapigenin in SGF_{sp}, FaSSIF and FeSSIF.

proof pharmaceutical quality of HMPs, they are a good method to characterize the biopharmaceutical quality of products during development and to control the quality of finished products. The problem of dissolution testing of HMPs in general is that often only selected constituents can be identified but not the hole spectrum of ingredients. On the other hand the guideline defines the extract in its entirety to be the active pharmaceutical ingredient.

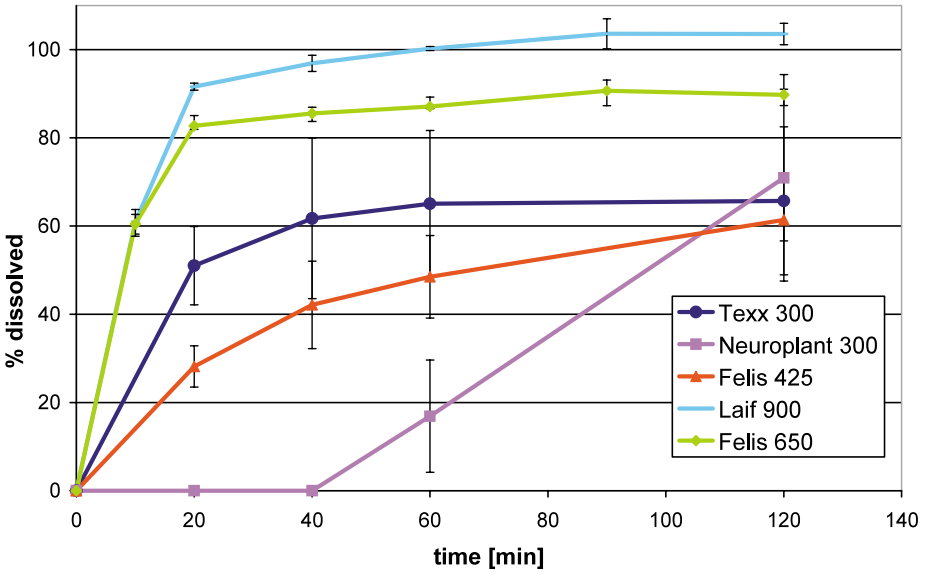

Fig. 4. Release of biapigenin in FeSSIF.

A previous study demonstrated that adequate release of the lipophilic hyperforin and hypericines was only obtained using FeSSIF as discriminating dissolution medium, whereas the dissolution of the flavonoids rutin, hyperoside, isoquercitrin and quercitrin is not depending on the medium [21]. Regarding the polarity biapigenin lays between the hydrophil flavonoids and the lipophil fraction. The dissolution profiles of the tested products show that biapigenin is released adequately in all of the three tested media. A release of approximately 70% was obtained in SGF_{sp}, FaSSIF and FeSSIF at least after 120 min test period. Our study together with earlier published data on dissolution behavior cover the dissolution profile of the wide spectrum of pharmaceutical active ingredients in SJW products. To guarantee adequate release of all pharmaceutical relevant ingredients dissolution tests with SJW products should be conducted in FeSSIF.

Investigating the release characteristics of biapigenin we could verify that each product has its individual dissolution profile. At that time there is a discussion in Germany about the "aut idem regulation" [22]. This regulation deals with the substitution in medication during treatment. Substitution is allowed when two preparations show essential similarity in their biopharmaceutical qualities. Essential similarity is guaranteed when two products are made of the same drug under the same manufacturing process using the same extraction solvent resulting in a comparable drug extraction ratio. Furthermore the application form and the daily dosage of extract have to be the same. It is important that the amount of pharmaceutical active ingredients is the same and that the release of the ingredients in the dissolution test are comparable. In our study we focused on the question if SJW products from different companies show essential similarity when they contain the same amount of SJW extract. The comparison of all tested products regarding their amount of biapigenin in each dosage form and their characteristic dissolution profile in FeSSIF allows the conclusion that the tested SJW products show no essential similarity. Data obtained from a clinical study of a certain SJW product cannot be used to verify clinical efficacy of a SJW product of a different manufacturer. Furthermore it is not appropriate to change the SJW medication in patients during treatment of depression.

References

[1] S. Kasper, Pharmacopsychiatry 34 (2001) 51–55.
[2] A. Nahrstedt, V. Butterweck, Pharmacopsychiatry 30 (Suppl. 2) (1997) 129–134.
[3] R. Berghöfer, J. Hölzl, Planta Med. 53 (1987) 216–217.
[4] D. Tekel'ová, M. Repcák, E. Zemková, J. Tótt, Planta Med. 66 (2000) 778–780.
[5] U. Simmen, J. Higelin, K. Berger-Büter, W. Schaffner, K. Lundstrom, Pharmacopsychiatry 34 (Suppl. 1) (2001) S137–S142.
[6] M. Wonnemann, Pharmacopsychiatry 34 (Suppl. 1) (2001) S148–S151.
[7] R. Scott Obach, J. Pharmacol. Exp. Ther. 294 (2000) 88–95.
[8] M. Gobbi, M. Moia, L. Pirona, P. Morazzoni, T. Mennini, Pharmacopsychiatry 34 (2001) 45–48.
[9] M. Nöldner, Therapieforum Biozentrum, 2001.
[10] M. Nöldner, Planta Med. 68 (7) (2002) 577–580.
[11] K. Westerhoff, Dtsch. Apoth. Ztg. 10 (2002) 1153–1175.
[12] J. Hölzl, E. Ostrowski, Dtsch. Apoth. Ztg. 127 (23) (1987) 1227–1230.
[13] S.G. von Eggelkraut-Gottanka, S. Abu Abed, W. Müller, P.C. Schmidt, Phytochem. Anal. 13 (2002) 170–176.
[14] M. Ganzera, J. Zhao, I.A. Khan, J. Pharm. Sci. 91 (3) (2001) 623–630.
[15] E. Galia, E. Nicolaides, D. Hörter, R. Löbenberg, C. Reppas, J.B. Dressman, Pharm. Res. 15 (5) (1998) 698–705.
[16] CPMP/ICH/281/95: Note for Guidance on Validation of Analytical Procedures: Methodology.
[17] K. Westerhoff, M. Wurglics, A. Kaunzinger, J.B. Dressman, M. Schubert-Zsilavecz, Dtsch. Apoth. Ztg. 142 (2002) 203–207.
[18] Biopharmaceutical Characterization of Herbal Medicinal Products, Pharm. Ind. 63 (10) (2001) 1005–1010.
[19] M. Wurglics, K. Westerhoff, A. Kaunzinger, A. Wilke, A. Baumeister, J.B. Dressman, M. Schubert-Zsilavecz, Pharmacopsychiatry 34 (2001) 152–156.
[20] Note for Guidance on specifications: test procedures and acceptance criteria for herbal drugs, herbal drug preparations and herbal medicinal products. CPMP/QWP/2820/00.
[21] K. Westerhoff, A. Kaunzinger, M. Wurglics, J.B. Dressman, M. Schubert-Zsilavecz, J. Pharm. Pharmacol. 54 (2002) 1–6.
[22] H. Blume, K.G. Brauer, T. Dingermann, E. Mutschler, I. Zündorf, Dtsch. Apoth. Ztg. 142 (10) (2002) 1205–1214.