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Development of a high-performance liquid chromatographic method with electrochemical detection for the determination of hyperforin

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Abstract

An HPLC method with electrochemical detection for the determination of hyperforin extracts without using additional sample precleaning has been developed and validated. The hyperforin solutions were separated isocratically using a mobile phase consisting of 10% ammonium acetate buffer (0.5 M, pH 3.7)–MeOH–acetonitrile (10:40:50, v/v) at a flow rate of 0.8 mL/min. Hyperforin was detected amperometrically with a glassy carbon electrode at a potential of +1.1 V versus Ag/AgCl/3 M potassium chloride reference electrode. Under these conditions, a plot of integrated peak area versus concentration of hyperforin was found to be linear over the range of 0.054–5.4 μg/mL, with a relative standard deviation of 2.2–8.6%. The limit of detection was 0.050 ng on column. The determination of the hyperforin content in a commercially available St. John's Wort preparation exhibited a mean content of 1.56 mg. Recovery experiments led to a mean recovery rate of 97 ± 5.8%. The proposed method is not time-consuming, sensitive and reproducible and is therefore suitable for routine analysis of hyperforin in herbal medicinal products.

Keywords: Hypericum perforatum; St. John's Wort; Pharmaceutical analysis; Hyperforin

1. Introduction

St. John's Wort extract preparations have been used for a long time as an effective alternative to synthetic antidepressants for treating mildly to moderately severe depressions. Although the mechanism of its antidepressant activity has not yet been completely elucidated, the antidepressant efficacy has been confirmed in most clinical studies. Despite the fact that *Hypericum perforatum* (St. John's Wort) ranks among the best-investigated plants, it has as yet not been possible to identify all the effective compounds of this "multi-substance-mixture". The extract contains a spectrum of different active pharmaceutical ingredients such as hyperforin (acylphloroglucinol-derivative), hypericins (naphthodianthrones), flavonol-glycosides, biflavones, procyanidines and phenylpropanes [1]. Hyperforin is the main source of pharmacological effects caused by the consumption of alcoholic extracts of St. John's Wort in the therapy of depression. It offers many effects comparable to that of synthetic antidepressants like the uptake inhibition of 5-hydroxy tryptamine, norepinephrine, and dopamine. But in addition the γ-aminobutyric acid and L-glutamate uptake is also inhibited. However, several studies indicate that flavones, e.g. rutin, and also the naphthodianthrones hypericin and pseudohypericin take part in the antidepressant efficacy [2–4].

Against this background it is reasonable—though not demanded by the approval authorities—to examine the pharmaceutical quality (e.g. batch-to-batch reproducibility, stability) of St. John's Wort extracts on the basis of their hyperforin (see Fig. 1) content.

Several HPLC methods with UV detection were developed for routine analysis of hyperforin in extracts or human plasma [5–21]. Furthermore, very sensitive LC–MS methods are suggested for the determination of hyperforin in *Hypericum perforatum* extracts [22,23] and for the investigation of pharmakokinetic behaviour in mouse brain [24].

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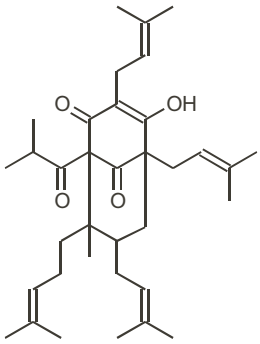

Fig. 1. Structure of hyperforin.

But LC–MS is generally not available for routine analysis. Beside these chromatographic methods a differential pulse voltammetric method based on oxidation of hyperforin has been established [25].

The oxidation of hyperforin at a glassy carbon electrode can be used for an electrochemical detection (ED) in connection with an HPLC method. On this basis we developed a new sensitive method for routine analysis of hyperforin in herbal medicinal products.

At first the chromatographic conditions as well as the amperometric settings at the glassy carbon electrode (e.g. oxidation potentials, pretreatment) were optimized. Then, as proof of principles, the developed HPLC–ED method was tested in a *Hypericum perforatum* containing herbal medicinal product. To verify the method presented herein, the same preparations were examined by means of an HPLC–UV method as to hyperforin and the results were compared with each other.

2. Experimental

2.1. Materials and reagents

Methanol and acetonitrile, both gradient grade, ammonium acetate p.a. and acetic acid of Suprapur quality were purchased from Merck (Darmstadt, Germany). Distilled water was purified with a Milli-Q Nanopure (Millipore, Bedford, MA, USA) system and was stored in Nalgene containers.

A mixture of hyperforin and adhyperforin in the form of the dicyclohexylammonium salt (Dr. Willmar Schwabe Arzneimittel, Karlsruhe, Germany) was used as standard. The hyperforin content was 62.39% according to the specification of the manufacturer. The tested tablets Abtei, containing 100 mg *Hyperici Herb. Extr. sicc*. are manufactured by Smith Kline Beecham (Herrenberg, Germany).

2.2. Instrumentation

The reversed-phase HPLC system consisted of a System Gold 125 Solvent module (pump; Beckman, Fullerton, CA, USA), a manual Rheodyne 7725i injection valve equipped with a 20 μL loop, and a programmable electrochemical detector 1049A (Hewlett-Packard, Vienna, Austria) with a glassy carbon working electrode and an Ag/AgCl/potassium chloride (3 M) reference electrode. The detector was set at +1.1 V versus Ag/AgCl/KCl. Data was recorded on a computer-based data system (System Gold Nouveau Software, Beckman).

For RP-chromatography a LiChroCart 125-4 Purospher RP-18 endcapped (5 μm) column (Hewlett-Packard) and a LiChroCart 4-4 LiChrospher 100 RP-18 (5 μm) guard-precolumn (Merck, Darmstadt, Germany) were used.

The mobile phase consisted of ammonium acetate buffer (0.5 M) adjusted to pH 3.7 with acetic acid (0.5 M)–MeOH–acetonitrile (10:40:50, v/v) and was filtered and degassed prior to use. The flow-rate was 0.8 mL/min. All separations were performed at room temperature (22 °C).

2.3. Standard and quality control solutions

Hyperforin stock solution (100 μg/mL): because of the high costs of hyperforin only 5 mg were available for our investigations. Therefore, 1.600 mg of the hyperforin salt should be weighed (considering the hyperforin content of 62.39%). In our case 1.731 mg of the salt were put into a 10 mL volumetric flask, dissolved in methanol and brought to volume. So this solution contained 108 μg hyperforin/mL and was stored at −18 °C. As a result of proper investigations concerning the stability of the stock solution it was found that there was no change in concentration for at least one month. Further standard solutions are prepared freshly each day by appropriate dilution of stock solution with methanol.

The same procedure was carried out for preparing hyperforin quality control solution using a different weighted sample. In our case the concentration of the quality control solution was 101 μg/mL.

2.4. Sample preparation for the determination of hyperforin in tablets

In order to eliminate inconstancy of weight, 10 coated tablets were finely pulverized in an analysis mill. An aliquot of this homogenized powder (461.2 mg corresponding to the average weight of one coated tablet) was placed in a 50 mL volumetric flask, taken to volume with ethanol–water (80:20, v/v) and allowed to extract for 10 min in an ultrasonic bath. The extract was filtered through a 40 μm syringe filter into brown glass vials and was frozen immediately (−18 °C). For analysis the defrosted extract was diluted with methanol in a ratio of 1:10 and 20 μL of this solution were injected into the HPLC system. Hyperforin is known to be highly photosensitive. Thus, all steps were performed protected from light by using amber glass utensils.

2.5. Linearity and limit of quantitation

For a long-term use of the analytical method a rigorous validation is indicated and requires the following procedures. For the preparation of calibration curve the stock solution was diluted freshly with methanol to obtain a set of six calibration standards (0.054, 0.135, 0.27, 0.54, 2.7 and 5.4 μg/mL). These standards were measured and the integrated peak areas were plotted against the corresponding concentrations of the injected standards. The complete procedure was repeated on six consecutive days. The so obtained six calibration curves were used to calculate a mean calibration graph. The limit of quantification was defined as that lowest concentration where an accuracy better than 20.0% was achieved [26].

2.6. Intraday and interday analysis using the quality control (QC) samples

For the purpose of quality control three different concentrations using a different stock solution (quality control solution) were prepared (quality control samples 0.38; 1.5 and 3.0 μg/mL). For the determination of the intraday precision and accuracy six replicates of the QC samples were analysed at the same day. The precision and the accuracy of the interday analysis were determined by analysing the QC samples on 6 different days.

3. Results and discussion

3.1. Optimization of the HPLC–ED system

In the course of optimization of the HPLC–ED system two different aspects have to be considered.

First the chromatographic conditions have to be adjusted carefully to obtain a sharp peak form corresponding to a high sensitivity and to obtain also a suitable retention time to avoid interferences with hydrophilic compounds in St. John's Wort extract. Both phosphate buffer and ammonium acetate buffer systems (in general used for electrochemical detection) were tested at pH 3.5–5.5. Higher pH values were not investigated, due to the fact that hyperforin is deprotonized at about pH 5.5 [25].

The second aspect regards the electrochemical detection. In this connection the oxidation potential was optimized to achieve a great rate with the oxidation process leading to an increase of sensitivity. Thus a hyperforin solution was injected at different potential settings in a range of +0.5 to +1.3 V versus Ag/AgCl/KCl. The electrochemical response versus oxidation potential is given in Fig. 2. As can be seen the maximum signal reached a plateau at potentials of +1.1 to +1.2 V. Since the background current and therefore the noise increases at higher potentials +1.1 V was used throughout this studies.

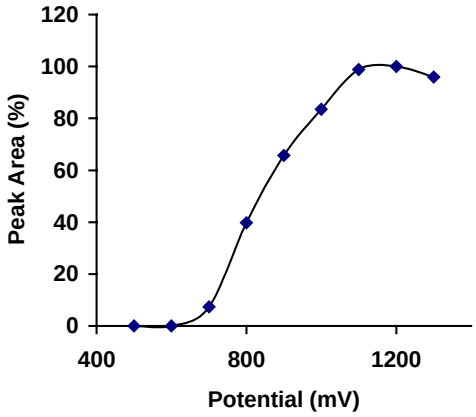

Fig. 2. Electrochemical responses (peak area) of hyperforin (54 ng) at various potential settings. The peak area at +1.2 V was arbitrary fixed as 100% value.

Both in chromatographic and in electrochemical respects best results with optimal sensitivity and reproducibility were obtained in NH_4Ac/HAc (pH 3.7; 0.5 M)–MeOH–acetonitrile (10:40:50, v/v) at a potential of +1.1 V. A typical chromatogram of hyperforin is shown in Fig. 3a. The retention time of hyperforin under these conditions was about 5.9 min. The smaller second peak (retention time about 6.8 min) is due to the content of adhyperforin.

3.2. Assay validation

Strict linearity between peak area and concentration of hyperforin in a range of 0.054–5.40 μg/mL (1.0×10^{-7} to 1.0×10^{-5} M) was observed. The "mean calibration curve" (six calibration curves; six measuring points per curve) produced the following linear equation: $Y = 8990 + 649454X$ (Y: peak area rate, X: μg/mL) ($R = 0.9998$). A summary of the precision and accuracy of the recalculated calibration samples is shown in Table 1. The rather moderate precision given there is, on the one hand, due to the fact that validation was carried out under a very strict standard procedure and on the other hand, that probably adsorption effects occurred at the working electrode [27,28].

Furthermore the precision and accuracy of the intraday and interday analysis were investigated on the basis of a set of quality control samples. The results given in Table 2 stand for a quite good trueness of the proposed method particularly considering intraday and interday analysis.

3.3. Limit of detection and limit of quantitation

The limit of detection (LOD) was obtained by successively decreasing the concentration of hyperforin as long as a signal-to-noise ratio of 3:1 appeared. The LOD was found to be 0.05 ng on column (volume of injection is 20 μL; corresponding to a concentration of 2.5 ng/mL). Following the procedure described in Section 2.5 the limit of quantitation (LOQ) was calculated to be 54 ng/mL.

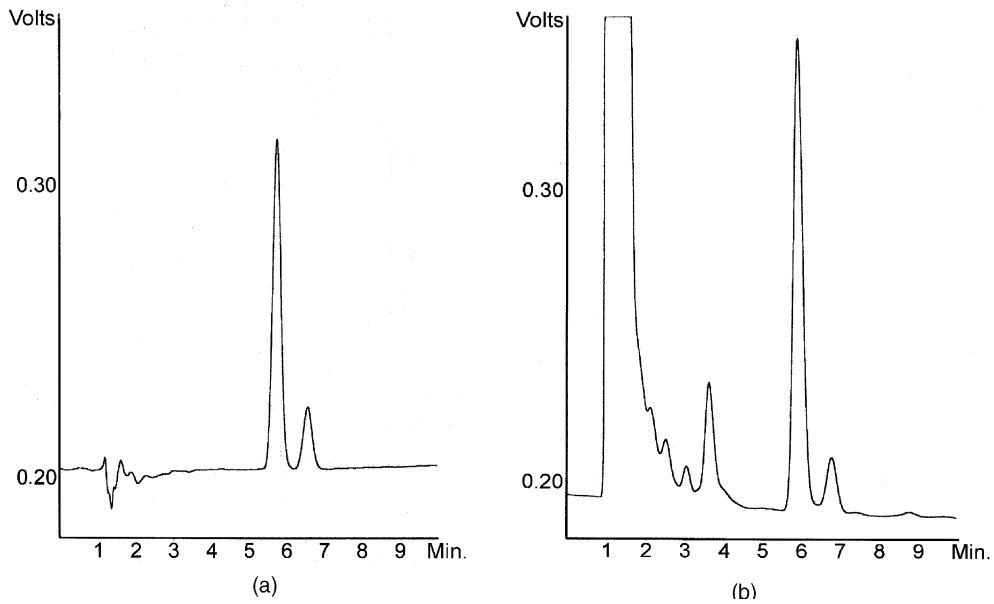

Fig. 3. Chromatograms of (a) hyperforin standard solution (injected amount 54 ng) and of (b) St. John's Wort extract preparation using ED at +1.1 V.

3.4. Quantitation of hyperforin in a St. John's Wort preparation

The hyperforin content of a commercially available St. John's Wort preparation was investigated. Following the sample preparation as described above for interday analysis ($n = 6$) a mean content of 1.60 mg hyperforin per tablet with a relative standard deviation (R.S.D.) of ±3.6% was obtained, intraday analysis ($n = 6$) yielded a mean content per coated tablet of 1.56 mg hyperforin with a R.S.D. of ±3.3%. A typical chromatogram of St. John's Wort extract is given in Fig. 3b. demonstrating no interference of the signal of hyperforin with other components in the extract. This indicates the specifity of the proposed method.

Recovery experiments (accuracy of matrix samples) were carried out by mixing the sample of St. John's Wort extract with a solution of known hyperforin content in a ratio of 1:1. These investigations ($n = 6$) resulted in a mean recovery rate of 97.0% and a relative standard deviation of ±5.8%.

Furthermore the presented method was compared with the up to now recommended HPLC method using UV detection [29]. The analysis of the same preparation applying these two methods led to the following results: 1.56 mg per tablet (HPLC–ED) and 1.42 mg per tablet (HPLC–UV; analysed in a laboratory in Frankfurt/Main, Germany). Thus it appears that the obtained results are in quite good agreement.

Table 1
Precision and accuracy of the recalculated calibration samples

Given (μg/mL)	Found, mean ± S.D. (μg/mL)	Precision (R.S.D., %)	Accuracy (percent deviation)
0.054	0.045 ± 0.0026	5.8	−16.7
0.135	0.128 ± 0.011	8.6	−5.2
0.27	0.262 ± 0.014	5.3	−3.0
0.54	0.549 ± 0.012	2.2	+1.7
2.7	2.732 ± 0.063	2.3	+1.2
5.4	5.383 ± 0.161	3.0	−0.3

Table 2
Intraday and interday precision and accuracy of the QC samples

Given (μg/ml)	Intraday		Interday	
	Precision (R.S.D., %)	Accuracy (percent deviation)	Precision (R.S.D., %)	Accuracy (percent deviation)
0.38	7.0	+9.7	8.9	+2.4
1.5	4.6	+4.9	5.4	+8.0
3.0	4.6	+2.0	5.5	+2.9

3.5. Conclusions

The need for quality assurance, including confirmation of the label strength and content uniformity has long been recognised even for herbal medicinal products. Although, in most herbal plants, with the exception of a few such as Silybum marianum and Piper methysticum, the pharmacologically active constituents are not yet known. In the case of *Hypericum perforatum* it has been well documented in the pharmacological literature that the lipophilic phloroglucine derivative hyperforin contributes to the antidepressant activity of the extract.

The proposed HPLC–ED method is appropriate not only for the determination of hyperforin in high dose preparations sold in pharmacies but also for preparations with a low dose of *Hypericum perforatum* extract sold in supermarkets. The presented method ranks if compared with the up to now recommended HPLC methods with UV detection among the most sensitive methods for determining hyperforin. Due to the high selectivity of electrochemical detection solid phase extraction or other sample precleaning methods are not necessary. The relative standard deviation of ±3.6% for the investigated St. John's Wort preparation indicates that the method is precise and reproducible.

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