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Single-drop liquid-phase microextraction for the determination of hypericin, pseudohypericin and hyperforin in biological fluids by high performance liquid chromatography

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Abstract

The analysis of hypericin, pseudohypericin (collectively called in this study hypericins) and hyperforin in biological fluids is reported using single-drop liquid-phase microextraction in conjunction with HPLC-UV-fluorescence detection. A new option for analysis of the active principle constituents in biological samples is proposed, reducing the steps required prior to analysis. There are several parameters which determine the mass transfer such as the extraction solvent, drop and sample volumes, extraction time and temperature, pH and ionic strength, stirring rate and depth of needle tip in the bulk solution. These parameters were chosen to optimize the performance in the current study. The method was validated with respect to precision, accuracy and specificity. The intra-day precision values were below 2.3% for the high concentration level of control samples and 6.2% for the low level. The respective inter-day precision values were calculated to be below 4.4 and 7.1%, respectively, for the two concentration levels. Accuracy of the method, calculated as relative error, ranged from −2.6 to 7.0%. It was demonstrated that as long as the extraction procedure is consistently applied, quantitative analysis is performed accurately and reproducibly in human urine and plasma samples. Limits of quantitation (LOQs) in urine were calculated to be 3, 6 and 12 ng/ml for pseudohypericin, hypericin and hyperforin, respectively. Slightly higher limits were measured in plasma, i.e. 5, 12 and 20 ng/ml, for the respective analytes.

Keywords: Pseudohypericin; Hypericin; Hyperforin; Analysis; Biological fluids; Single-drop liquid-phase microextraction

1. Introduction

St John's wort (*Hypericum perforatum* L.) has been known since antiquity for many medicinal properties such as hepatic disorders and gastric ulcers. In the last two decades, anti-inflammatory [1], anti-microbial [2], anti-viral [3], anti-depressant [4] and cytotoxic [5] activities have also been attributed to the total extract or individual components.

In recent years, increased interest in hypericin, one of the major components of the plant, as a potential photosensitizing anticancer agent has arisen. Several studies established the powerful in vivo and in vitro antineoplastic activity of hypericin in the absence of or upon irradiation [6,7]. Associated experimental results suggest that hypericin has considerable potential for use as a sensitizer in the photodynamic therapy of cancer [8,9]. Recently, also, the possibility of using hypericin as a diagnostic tool for the fluorescence detection of flat neoplastic lesions in urine bladders has been investigated [10].

Anti-depressant applications of St John's wort medicinal products (e.g. Psychotonin®, Neuroplant®, Hyperforat®) have become increasingly popular in Europe, particularly in Germany, where physicians routinely prescribe herbal medicines. The anti-depressant activity was first attributed to hypericin, its derivatives and polyphenols flavonols [11,12], but recent pharmacological and clinical results focus on hyperforins, as the main active ingredients of the extract

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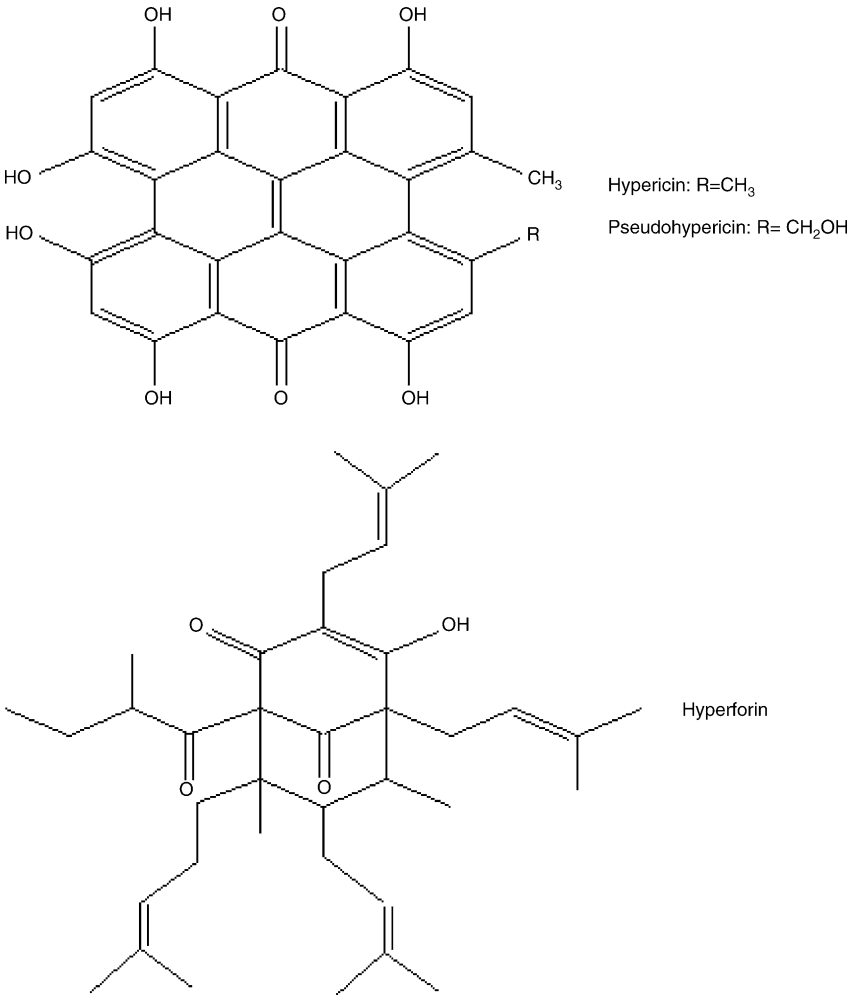

Fig. 1. Chemical structures of hypericin, pseudohypericin and hyperforin.

[13,14]. Thus, the standardisation of the extracts based on hypericin can no longer be proposed as a tool to evaluate potential benefits or risks of St. John's wort preparations. Jones et al. found that during a routine drug history, one in seven patients did not disclose that they were taking herbal medicines [15]. In another study, half of the outpatients reported that their doctor or pharmacist was unaware that they were taking St John's wort [16]. However, detailed information about the concurrent drug use is important because exposure to unknown drugs may hamper individualization of therapy and drug safety [17].

St. John's wort extracts are prescribed not only as herbal medicinal products but also as a top-selling botanical dietary supplement both standardised using the naphthodianthrones of the hypericin group, calculated as 0.2–1 mg hypericin daily dose. Finally, St. John's wort preparations have recently been used as an ingredient in some food products sold as functional foods [18].

A multitude of methods have been developed for the measurement of hypericin, pseudohypericin and hyperforin (Fig. 1). Some of them have been reported in the use, in a variety of biological media [19–27]. The methods employed hitherto in such matrixes require apolar organic solvents where hyperforin is unstable. A pretreatment step, most frequently solid-phase extraction, for clean up and preconcentration is necessary in order to detect low concentration levels.

During the last 10 years, with the upsurge of miniaturization in analytical chemistry several liquid–liquid extraction alternatives drew the attention of researchers. The major incentive behind this has been to speed up extractions, reduce the consumption of organic solvents and to facilitate towards automation. Liquid-phase microextraction, performed by using either a single drop of solvent [28–32] or a small length of porous hollow fiber-protected solvent [33], has shown to be an attractive alternative for sample preparation. In one of the single-drop modes, the so-called single-drop liquid-phase microextraction (SD-LPME), the organic micro droplet is placed into the aqueous sample and the analytes are extracted into the organic droplet (microextract) based on passive diffusion. It was reported that SD-LPME has comparable extraction efficiency and reproducibility with the widely used solid-phase microextraction.

Prompted by the advantages of SD-LPME, herein we report on the analysis of hypericin, pseudohypericin (collectively called in this study hypericins) and hyperforin in biological fluids using SD-LPME, in conjunction with HPLC. The primary purpose is to propose a new option for analysis of the active principle constituents in biological samples, reducing the steps required prior to analysis, without compromising, at the same time, the sensitivity. The solvents used in SD-LPME scheme after convenient dilution with methanol, are compatible with reversed-phase HPLC used to separate the analytes considered.

2. Experimental

2.1. Reagents and samples

Hypericin, pseudohypericin and hyperforin were purchased from Alexis Corp. (Lausen, Switzerland). Stock solutions of 0.1, 2.0 and 0.05 mg/ml in methanol were prepared under sonication, for pseudohypericin, hypericin and hyperforin, respectively. Solutions were stored at −5 °C in aliquots of 0.1 ml, while the bulks of the stock solutions were maintained at −18 °C. Sodium phosphate, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium hydroxide used for the preparation of the buffer solutions were from Riedel-de Haën (puriss p.a.). The same supplier was for HCl (37%, puriss p.a.) and sodium chloride (puriss p.a.). HPLC-grade methanol, hexane, toluene, ethyl acetate and chloroform were from LabScan (Labscan Ltd., Dublin, Ireland) while *n*-octanol (99%) was from Sigma (St. Louis, MO, USA). Aqueous samples were prepared by spiking double distilled water with the analytes at known concentrations (50, 100 and 150 ng/ml for pseudohypericin, hypericin and hyperforin, respectively) to study extraction performance under different conditions.

Urine samples were collected from laboratory personnel that were taking occasionally *H. perforatum* L. extracts for the purposes of the present study. Samples were filtered before use in SD-LPME to remove suspended particles.

Blood was collected directly in vials containing EDTA as anticoagulant from healthy volunteers who received concentrated ethanolic extract of *H. perforatum* L. received from 10 g of plant. After centrifugation at 1500 × *g* for 15 min, the clear plasma supernatant was collected and stored at −18 °C, until the moment of use. Frozen, drug-free plasma for calibration curves was obtained from the University Hospital and thawed at room temperature before use.

The *H. perforatum* extracts where obtained by extracting with ethanol the plant collected during May 2002 from the region of Epirus (Greece).

2.2. Single-drop liquid-phase microextraction

One 10-μl microsyringe with a bevel needle tip (Hamilton, Reno, NV, USA) was used for introducing organic drop in the sample. Single-drop LPME consists of the following steps: (1) sample solution is agitated with a magnetic stirrer by means of a 10 mm × 3 mm stir bar; (2) the microsyringe is rinsed with the organic solvent for several times to ensure that no air bubbles are left in the barrel and the needle; (3) a specified volume of organic solvent is drawn into the syringe and with the needle tip out of the solution, the plunger is depressed by 1 μl; (4) the needle, fixed with a stand and clamps, is then inserted through the septum of the sample vial (10-ml capacity) and immersed in the sample; (5) the plunger is pushed down to expose the organic drop to the stirred aqueous solution for a preset period of time; (6) the drop is retracted into the microsyringe, which in turn is removed from the sample vial; (7) the organic solvent drop is transferred to a micro-vial and made up to 30 μl with methanol; (8) 20 μl is injected into the HPLC by means of a 50-μl microsyringe with a flat-cut needle tip (glass barrel, I.D. 0.46 mm, needle I.D. 0.11 mm) (Hamilton, Reno, NV, USA).

2.3. HPLC assay of hypericin, pseudohypericin and hyperforin

Liquid chromatographic analysis of hypericin and pseudohypericin was performed on a Shimadzu HPLC system (Duisburg, Germany) consisting of a pump LC 10AD, a 20-μl sample loop, a manual Rheodyne injector (7725i, Cotati, CA, USA), a column heater CTO 10A and a fluorescence detector RF 551. Class LC10 software Version 1.6 (Shimadzu) was used for data analysis and processing. Hypericins were eluted isocratically, at 30 °C, on a reversed-phase Hypersil C_{18} column (ThermoFinnigan, San Jose, CA, USA) protected by a guard column of the same material and quantified by fluorescence detection at 322/593 nm (ex/em). The mobile phase was prepared weekly by mixing 95 volumes of methanol with 5 vol. of phopshate buffer solution (pH 2.2). For the preparation of the buffer solution, 2.5 g of KH_2PO_4 was dissolved in 950 ml double distilled water, adjusted to a pH of 2.2 with concentrated phosphoric acid and filled up to 1000 ml with double distilled water. The mobile phase was filtered before use, through a 0.45-μm nitrocellulose membrane and was delivered isocratically at a flow-rate of 1 ml/min.

Hyperforin was chromatographed on the same HPLC system furnished with a spectrophotometric detector SPD 10AV at 276 nm, connected in series with fluorescence detection, as per conditions for hypericins. All the analytes were quantified using peak heights.

The total chromatographic analysis time per sample was 6 min. No column wash-out step between injected samples was required, as the strength of the mobile phase (95% methanol) was sufficiently high to remove the extraneous peaks of the sample matrix.

2.4. LC–ESI/MSD ion trap-identification

The LC–MSD-trap-SL" system was an Agilent Technologies (Palo Alto, CA, USA) was equipped with an electrospray

interface operating in positive ionization mode, an autosampler and autoinjector. The operating conditions were: accumulation time, 300 ms; dry temperature, 350 °C; capillary voltage, 3500 V; nebulizer, 40 psi; dry gas, helium at 12 l/min. Ion trap full scan analysis was conducted from m/z 50 to 700 with an upper fill time of 200 ms. A 2-μl sample volume was injected. Complete system control and data evaluations were done on the HP ChemStation for LC/MS.

The analytical column was Zorbax® SB-C18 (2.1 mm × 30 mm I.D., 3.5 μm) from Agilent Technologies (Palo Alto, CA, USA). The temperature of the column was 30 °C and the separation program was isocratic with 95:5 MeOH/ammonium formate 5 mM.

2.5. Sample preparation for hypericins and hyperforin in human urine and plasma

2.5.1. Urine

In a 10-ml aluminum-wrapped glass vial, 4.5 ml of filtered urine was mixed with 0.5 ml buffer phosphate 0.1 M, pH 6.0.

2.5.2. Plasma

In a 5-ml aluminum-wrapped sample vial, 1 ml plasma was mixed with 2 ml methanol and the sample was spun for 3 min at 1300 × g. From the supernatant, 1 ml was transferred to a 10-ml aluminum-wrapped glass vial and vortex-mixed subsequently with 3.5 ml double distilled water and 0.5 ml buffer phosphate 0.1 M, pH 6.0.

The LPME procedure was applied for both matrixes, as detailed under Section 2.2.

2.6. Quantification and quality control samples

Calibration standard solutions were prepared at concentrations up to 290 ng/ml by proper dilutions of stock solutions in the tested matrixes (i.e. double distilled water, human urine and plasma) instead of evaporating volumes to dryness and reconstituting in the target matrixes. For the preparation of quality control samples appropriate aliquots of the hypericin, pseudohypericin and hyperforin stock solutions were added to blank human urine and plasma at two different concentration levels; the first one three times the limits of quantitation for each compound and the other one ten times these values, for the respective analytes. Calibration standards, blank urine and plasma samples and quality control samples were stored in aliquots of 250 μl at −18 °C until analysis.

3. Results and discussion

3.1. Optimization of single-drop liquid-phase microextraction parameters

The initial objective was to optimize the SD-LPME sampling conditions and to fix the parametric values for the extraction of hypericin, pseudohypericin and hyperforin. The dynamic characteristics of the microextraction process are closely related to the mass transfer of the analytes from the aqueous to the organic phase. Intrinsically, the LPME process is driven by the difference-gradient of concentration between aqueous and organic phases. There were several parameters which determine the mass transfer such as the extraction solvent, drop and sample volume, extraction time and temperature, pH and ionic strength, stirring rate and depth of needle tip in the bulk solution. These parameters were chosen to optimize the performance in the current study via a univariate optimization approach. The chromatographic peak height was used to evaluate the extraction efficiency under different experimental conditions.

If one wants to avoid evaporation of the solvent and reconstitution in the corresponding matrix, the presence of organic solvent especially in the optimization experiments seems inevitable, as hypericin and pseudohypericin are scarcely soluble in any other solvent except methanol. This was advocated by the fact that methanol up to 16% in the bulk extraction aqueous solutions and samples, does not provoke any extraction difficulties nor does it diminish the extraction yield. A momentary undesirable bubble formation can be overcome by a 1-min vigorous agitation of the sample, before extraction.

3.1.1. Nature of microdrop organic solvent

In SD-LPME, equilibrium of solute is developed between two immiscible liquid phases: the aqueous and the organic. In line with this practice, in our study, solutes were extracted from an aqueous solution into an immiscible organic solvent. Toluene, hexane, chloroform, ethyl acetate and *n*-octanol differing in physicochemical properties (i.e. polarity, surface tension, water solubility, etc.) were considered for the extraction of the analytes. Hypericins are insoluble in water, slightly soluble in polar organic solvents and completely insoluble in apolar solvents. Traditionally, methylene chloride (density 1.33 g/ml, solubility 1.32 g/100 ml), ethyl acetate (density 0.894 g/ml, solubility 0.8 g/100 ml) and chloroform (density 1.49 g/ml, solubility 0.795 g/100 ml) are used in classical LLE to extract them. Hyperforin is lipophilic but decomposes quickly in non-polar reagents [34,35]. Notwithstanding the fact that the apolar toluene and hexane are used in SD-LPME and are polarity-compatible with hyperforin giving high extraction efficiencies, they are inappropriate for our system, for the above-mentioned reason. Moreover, these solvents are proved not to be the most suitable for SD-LPME because of the difficulty to be held as microdrops at the tip of the microsyringe for a certain time length (≥15 min) due to their low viscosity and density. Ethyl acetate, chloroform and hexane in a drop-based extraction for the three analytes of concern, provided decreasing extraction yield in the order mentioned. In contrast, *n*-octanol, being practically insoluble in the water for a restricted extraction period, was suitable for both hypericins, but less preferential for hyperforin. Besides, drop dislodgement occurs as *n*-octanol, with density 0.83 g/ml, in a biphasic system with water tends to

form the upper layer. The overall situation is vastly improved by incorporating into *n*-octanol a small volume of the heavier and less polar chloroform. In a range of 10–30% with respect to chloroform, a slight decline in the extraction yield of hypericins is accompanied by a more striking augmentation of hyperforin yield and drop stability. Accordingly, an *n*-octanol:chloroform mix, at a ratio 7:3 (v/v) was chosen as the organic solvent for extracting all three constituents of interest in the single drop.

3.1.2. Microdrop and sample volumes

The amount of the analytes extracted into an organic drop is linearly proportional to the drop size at equilibrium, as depicted by the following equation [36].

$$N = KV_{\mathrm{org,eq}}C_{\mathrm{aq,in}} \quad (1)$$

where N is the number of moles of analytes extracted by the organic drop; K is the distribution coefficient of an analyte between the aqueous phase and the organic drop; $V_{\mathrm{org,eq}}$ is the volume of organic drop at equilibrium; and $C_{\mathrm{aq,in}}$ is the initial concentration of the analyte in aqueous solution. It was demonstrated that a linear increase in HPLC signals occurs with the size of *n*-octanol-chloroform in the range of 1–3.5 μl, as predicted from Eq. (1). Importantly enough, this increase is striking in the case of hypericins; the contrary holds for the hydrophobic hyperforin due probably to partial dissolution of chloroform in the aqueous bulk solution in the course of time, a phenomenon further stimulated by stirring. While in aqueous standard solutions drop volumes up to 3.5 μl can remain attached to the needle for time periods longer than half an hour, in urine and plasma, on account of the matrix composition and the presence of fine particles, drop volumes higher than 2 μl are prohibitedly large and they finally dislodge from the needle of the microsyringe. We finally opted for a 2-μl drop size for all the extractions, as this volume gave the highest potential for good enrichment under these experimental circumstances.

Extractions from sample volumes of 3, 5, 7 and 9 ml were performed and a total sample volume of 5 ml was selected to reconcile low consumption of biological sample and uncompromised chromatographic signal.

3.1.3. Extraction time and temperature

The SD-LPME is a process dependent on equilibrium rather than exhaustive extraction. In most SD-LPME applications, the efficiency of extraction increased with extraction time. The extraction of the three analytes into the organic drop and the dissolution of some of drop into the aqueous solution govern the concentration in the microdrop. Again, the factor of chloroform dissolution was introduced. Loss was largely due to drop depletion at long contact time. However, a certain period of time was needed for the equilibrium between organic drop and aqueous phase to be established. It was demonstrated that extraction time exerts strong influence on the peak heights. The amount of analytes extracted

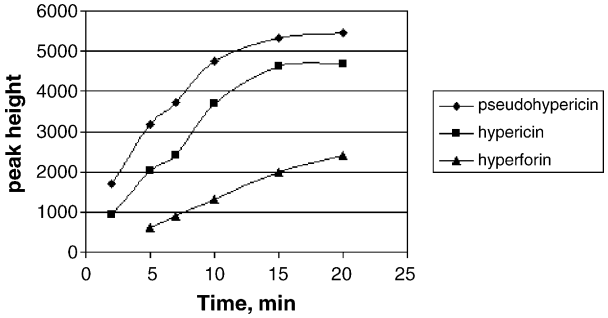

Fig. 2. Time dependence of the equilibration of the hypericin, pseudohypericin and hyperforin between the aqueous and drop-based phase. Extraction conditions: temperature, 40 °C; organic solvent, *n*-octanol:chloroform 7:3 (v/v); drop volume, 2 μl; pH 6.0; NaCl: 0%; stirring rate, 150 rpm; sampling depth, 1.5 cm.

should increase with longer extraction time until a maximum is attained at equilibrium. It was found from the curves visualized in Fig. 2, that signal kept rising linearly in the first 10 min, after which it roughly flattened out. It was indicated that the equilibration conditions were reached after about 20 min, but the change is not great to warrant the selection of exposure times longer than 15 min, a time length that additionally maintain better sample throughput. In addition, it is not necessary to reach equilibrium provided that the extraction conditions are reproduced.

Temperature was found to be critical for the extraction of all three analytes (Fig. 3). The higher the temperature the better the extraction efficiency achieved. On the other hand, temperatures of 45 °C cause the solvent drop to be unstable due to bubble formation in the bulk solution. Still, the inevitable evaporation of chloroform and depletion of drop are compensated for by the high extraction efficiency, as a result of the increased extraction yield. In this context and considering the rather short extraction time employed (15 min) along with the fact that hyperforin is temperature-sensitive, we were able to attain an unimpeded SD-LPME process at 40 °C following a step of vigorous agitation prior to extraction.

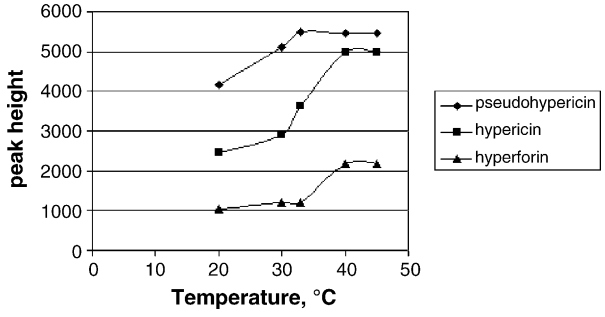

Fig. 3. Temperature dependence of the equilibration of analytes between the aqueous and drop-based phase. Extraction conditions: extraction time, 15 min; organic solvent, *n*-octanol:chloroform 7:3 (v/v); drop volume, 2 μl; pH 6.0; NaCl: 0%; stirring rate, 150 rpm; sampling depth, 1.5 cm.

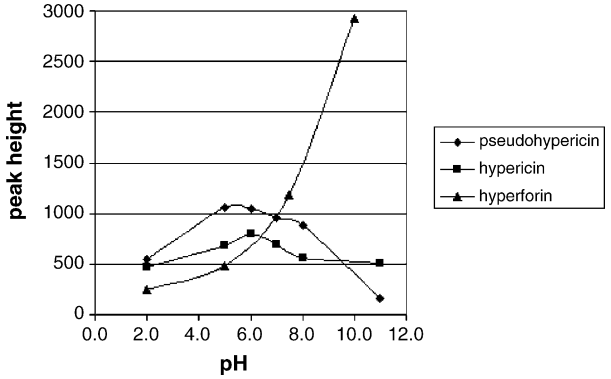

Fig. 4. Effect of aqueous solution pH on the extraction of analytes in the drop, as expressed by respective peak heights. Extraction conditions: extraction time, 15 min; temperature, 40 °C; organic solvent, *n*-octanol:chloroform 7:3 (v/v); drop volume, 2 μl; NaCl: 0%; stirring rate, 150 rpm; sampling depth, 1.5 cm.

3.1.4. pH and ionic strength of the test solution

The pH of the extracted solution is expected to induce significant impact on the extraction. In order to examine the extent to which this parameter influences the system, experiments were carried out in original aqueous solutions containing all three analytes and properly varying the pH in the range from 2 to 11, with either 1N NaOH or 1N HCl. After microdrop extraction, the sample pH was checked and found to sustain its original value. Fig. 4 represents the effect of pH on the extraction. It is important that while peak heights were almost doubled for hypericins when going from pH 2 to 6 and then recover their initial values in alkaline ambience, a dramatic augmentation of hyperforin signal stimulates further examination. The markedly increased peak, appearing as hyperforin at basic medium, has a UV–vis spectrum identical to that of hyperforin. It is known that hyperforin is very sensitive to oxidation and susceptible to photodegradation whilst in basic medium decomposes completely [35]. (It was, therefore, imperative to protect the samples during the process period at the bench by carefully wrapping the sample vials housing the samples with aluminum foil following a rapid step of preparation for SD-LPME). It was not until very recently that a work was focused on the extensive study of hyperforin degradation products [37]. Adhyperforin, being one of them, is eluted in the chromatogram well resolved from hyperforin (retention time: 6.28 min) and therefore peak assignment to adhyperforin in basic medium should be ruled out. Considering the unequivocal instability of hyperforin as solution pH increases, we can presume that this curious signal increase is attributed to furohyperforin and/or furohyperforin analogue, which are major degradation compounds and their formation is favored at high pH values. Our assumption is further validated by the mass spectrum received (Fig. 5), wherein the base peak at *m/z* 553.5 and the fragment at *m/z* 485.4 are characteristic of such an analogue [37]. A pH value of 6.0 was the reasonable compromise for the extraction and stability of all the analytes.

The increased ionic strength of the sample solution is expected to decrease the water solubility of the analytes (salting-out effect) and consequently to enhance the extraction yield. It is worth mentioning that recorded was the opposite by several researchers for SD-LPME, which was consistent with our results [38,39]. Fig. 6 shows the influence of salt addition (NaCl) on the extraction efficiency. It is obvious that salt, at any concentration, deteriorated extraction efficiency, more pronouncedly in the case of hyperforin and pseudohypericin. The NaCl dissolved in water might have changed the physical properties of the Nerst diffusion film and reduced the rate of diffusion of the target analytes into the drop. This signifies that with increased salt concentration the diffusion of analytes towards the organic drop becomes more and more difficult limiting thus extraction. The aforementioned behavior of the studied system negates the need for salt addition.

3.1.5. Stirring rate

Stirring rate reduces the necessary time to reach thermodynamic equilibrium and thus increases extraction efficiencies.

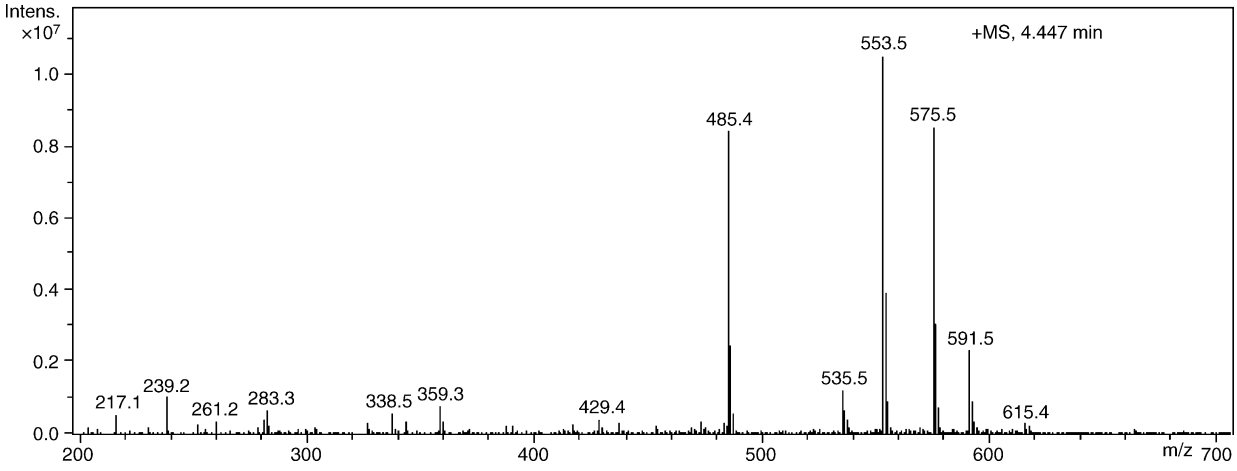

Fig. 5. Mass spectrum of the furohyperforin analogue by LC/ion trap MS detection.

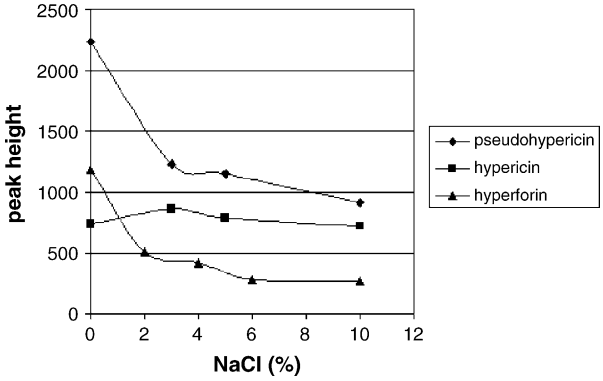

Fig. 6. Effect of addition of NaCl on the peak heights. Extraction conditions: extraction time, 15 min; temperature, 40 °C; organic solvent, *n*-octanol:chloroform 7:3 (v/v); drop volume, 2 μl; stirring rate, 150 rpm; sampling depth, 1.5 cm.

The diffusion coefficient in the aqueous phase increases with increasing stirring rate because faster agitation can decrease the thickness of the diffusion film in the aqueous phase [40]. This film theory was substantiated to be valid in the SD-LPME method [41]. Extraction efficiency for hypericins and hyperforin increased with higher stirring speed in concurrence with the results of other researchers. However, stirring speeds higher than 200 rpm gave rise to destabilization of the organic drop, whilst over 300 rpm drop detachment was almost instant, increasing the potential of formation of air bubbles and drop depletion, as well. From the above line of thinking, a stirring rate of 150 rpm was fixed for further microextractions.

3.1.6. Sampling depth

The last step was to optimize the distance between the needle tip and stirring bar. Positioning the needle in the aqueous phase at a fixed height with stands and clamps, could reasonably improve the precision of the method. It was found that the extraction yield was invariably the same in the studied range, between 0.5 and 3.0 cm from the stirring bar, for all the analytes of concern, albeit the drop being more markedly unstable at 0.5 cm. Although precision was found not to be risked with small alterations of sampling depth, the needle tip was finally positioned at the fixed height of 1.5 cm above the stirring bar to avoid unforeseen circumstances during the realization of the experiments.

3.2. Liquid chromatographic separation

As gradient method takes longer time to run than an isocratic method, because of the need for column equilibration after each run, an isocratic separation was first attempted. A 95% MeOH in the mobile phase ensures a rapid analysis and demonstrates resolution. Fig. 7 shows the chromatograms with hypericins and hyperforin of (A) fortified plasma (pseudohypericin, 25 ng/ml; hypericin, 20 ng/ml; hyperforin, 60 ng/ml), (B) fortified urine sample (pseudohypericin, 50 ng/ml; hypericin, 90 ng/ml; hyperforin, 30 ng/ml), (C) plasma and (D) urine after administration of hypericum extract. Hypericin and pseudohypericin were well separated under the HPLC isocratic conditions applied. Retention times were 2.42 min for pseudohypericin and 3.16 min for hypericin, in fluorescence detection. Fluorescence detection has a serious sensitivity advantage over UV–vis detection of hypericins. Hyperforin, with retention time of 5.70 min in UV–vis detection under identical chromatographic conditions, was well separated from other peaks. No interferences in the respective retention times were observed in blank urine and plasma samples. Late-eluting endogenous species were removed without necessitating the use of a gradient method to help get rid of these interferences.

3.3. Method validation

3.3.1. Analytical performance

The validation of a method is a process to establish that the analytical performance parameters are adequate for their intended use. The optimized extraction protocol was aimed to be applied to the analysis of human urine and plasma samples. Since hypericins and hyperforin are not endogenous substances, there were real blank biological samples available. Comparison of extraction from the same matrix showed no statistically significant differences in the slope values ($\alpha = 0.05$) for multiday calibration studies. The slopes of three curves prepared on three different days for the three analytes have coefficients of variation (CVs) less than 5%. However, slopes among the different matrixes are not uniform – lower slope values are noted for urine and even lower for plasma – signifying the need to use calibration curves in the respective matrixes for real sample analysis. The relationships between peak height and amount of the compound added to aqueous, urine and plasma matrix were always linear with square correlation coefficient exceeding invariably 0.9965. Linearity of the calibration graphs in biological matrixes extents up to 130, 200 and 290 ng/ml for pseudohypericin, hypericin and hyperforin, respectively. The intra-assay and inter-assay precision (expressed as CV%) and the accuracy of the method (expressed as %bias) were assessed analyzing three replicates of urine and plasma quality control samples at two different concentration levels; the first one three times the limits of quantitation for each compound and the other one ten times these values. Estimates of the intra- and inter-day precision were afforded by performing a one-way analysis of variance (ANOVA) with day as grouping variable. A one-tailed *F*-test was carried out to test whether the mean squares differed significantly. The data indicated that the assay method is reproducible within the same day (intra-assay) and within different days (inter-assay) and the precision could be calculated. The intra-day precision values were below 2.3% for the high concentration level and 6.2% for the low level. The respective inter-day precision values were calculated to be below 4.4 and 7.1%, respectively, for the two concentration levels.

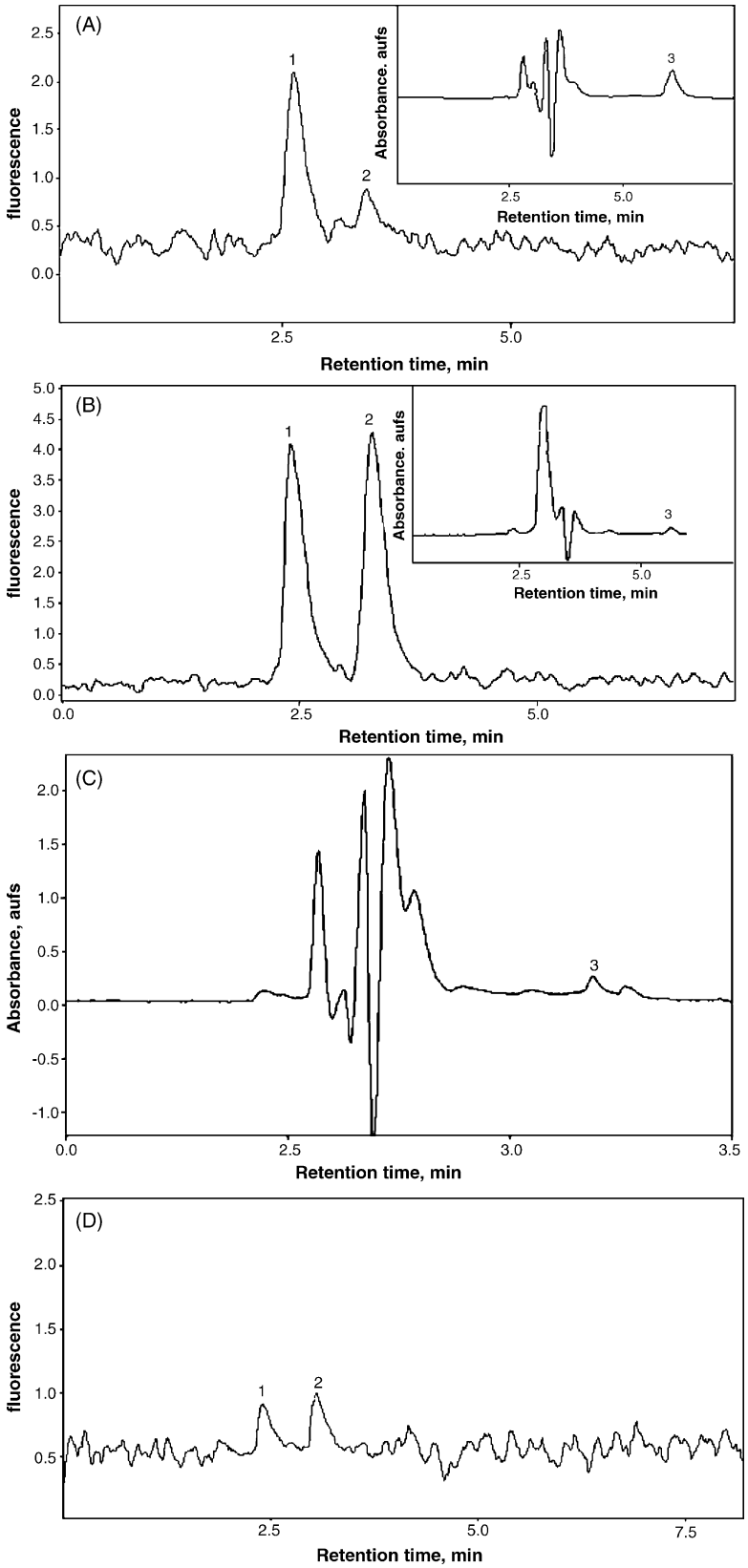

Fig. 7. HPLC traces of extraction from (A) spiked plasma (pseudohypericin, 25 ng/ml; hypericin, 20 ng/ml; hyperforin, 60 ng/ml) using fluorescence detection, (B) spiked urine (pseudohypericin, 50 ng/ml; hypericin, 90 ng/ml; hyperforin, 30 ng/ml) using fluorescence detection, (C) plasma 5 h post administration using UV detection, (D) urine 12 h post administration using fluorescence detection. The insets show the HPLC-UV chromatograms of the respective extracted samples. Peak assignment: (1) pseudohypericin, (2) hypericin and (3) hyperforin.

Accuracy of the method (%bias) in urine and plasma was calculated as relative error [(measured concentration − nominal value)/nominal value × 100]. Values ranged from −2.6 to 7.0% with the highest one corresponding to the low spiking level.

As long as the extraction procedure is consistently applied, quantitative analysis is performed accurately and reproducibly.

Limits of quantitation (LOQs) – calculated as concentrations giving signal-to-noise ratio = 10 – were sufficiently low in urine and plasma. In urine, 3, 6 and 12 ng/ml were measured for pseudohypericin, hypericin and hyperforin, respectively. Slightly higher limits were measured in plasma, i.e. 5, 12 and 20 ng/ml, for the respective analytes.

3.3.2. Applications

An attempt to implement the microextraction to untreated plasma was unsuccessful because the drop shortly after formation became turbid and was rendered unable to extract even low amounts of the analytes. We tried to circumvent the deproteinization step by simply diluting the plasma sample by 1:5 with buffer solution, pH 6.0, prior to extraction step. Nevertheless, the mentioned problem of limiting extraction yield was still encountered. Apparently, removal of proteins from the sample was a prerequisite for the accurate and sensitive detection of hypericins and hyperforin, at nanogram/liter levels. Protein precipitation techniques have been reported for the accurate assay of the analytes in biological samples. The widely used protein precipitation reagents include, organic solvents (acetonitrile, methanol), acids (sulfosalicylic, perchloric and trichloroacetic acid) etc. Acids increase the ionic strength and could readily change the pH of the sample. As microextraction can withstand methanol in the bulk extraction solution, it was that solvent which was selected for deproteinization. Chromatograms showed non-detectable levels of hypericins in plasma 5 h post administration; in contrast, hyperforin, at a concentration of 0.055 μg/ml was measured (Fig. 7C). The chromatographic retention times and UV–vis spectra of analytes in the extracted matrix sample matched well with those of standard solutions thus confirming specificity.

Urine was subjected to drop-microextraction without any pretreatment. Urine concentrations were monitored over 12 h post administration of hypericum ethanolic extract. It has been reported that hypericin and pseudohypericin are not detectable in urine most likely due to conjugation with glucuronic acid and subsequent excretion into the bile [42]. At 2 and 7 h post administration, no detectable amounts of hypericins and hyperforin were observed. Yet, after 12 h, visual inspection of the extracting drop revealed a fade reddish color indicative of the presence of hypericins in the urine of the subjects. In contrast to previous findings, HPLC analysis of the drop resulted in the detection of hypericins albeit at concentrations lower than LOQ.

Using the extraction and analysis protocol developed for urine and human plasma, a complete analysis can be performed within 22 and 25 min, respectively. This allows two samples per hour to be analyzed if the extraction and separation are run in tandem. Maximum throughput is attained by simultaneously run both extraction and separation.

4. Conclusion

We have developed and introduced a method to suit the requirements of clinical studies involving hypericum perforatum extract that allows a rapid, inexpensive and reproducible determination of low concentrations of hypericin, pseudohypericin and hyperforin in human plasma and urine. The drop-based preconcentration methodology combined with isocratic reversed-phase HPLC, offers an effortless and selective means of monitoring urine and plasma levels in clinical samples. The system is flexible and amenable to improvements such as incorporating detection by mass spectrometry or using micro-HPLC systems compatible with the low-volume requirements of microextraction, towards improving identification and LOQs. Because of the sensitivity, the ease of use and simplicity, the method can be used routinely for screening purposes.

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