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Identification of the major constituents of *Hypericum perforatum* by LC/SPE/NMR and/or LC/MS

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Abstract

The newly established hyphenated instrumentation of LC/DAD/SPE/NMR and LC/UV/(ESI)MS techniques have been applied for separation and structure verification of the major known constituents present in Greek *Hypericum perforatum* extracts. The chromatographic separation was performed on a C18 column. Acetonitrile-water was used as a mobile phase. For the on-line NMR detection, the analytes eluted from column were trapped one by one onto separate SPE cartridges, and hereafter transported into the NMR flow-cell. LC/DAD/SPE/NMR and LC/UV/MS allowed the characterization of constituents of Greek *H. perforatum*, mainly naphtodianthrones (hypericin, pseudohypericin, protohypericin, protopseudohypericin), phloroglucinols (hyperforin, adhyperforin), flavonoids (quercetin, quercitrin, isoquercitrin, hyperoside, astilbin, miquelianin, I3,II8-biapigenin) and phenolic acids (chlorogenic acid, 3-*O*-coumaroylquinic acid). Two phloroglucinols (hyperfirin and adhyperfirin) were detected for the first time, which have been previously reported to be precursors in the biosynthesis of hyperforin and adhyperforin.

Keywords: *Hypericum perforatum*; LC/SPE/NMR; Dereplication; Hyperforin; Hyperfirin; Flavonoids

1. Introduction

Hypericum perforatum, commonly named St. John's wort in Western Europe, is an herbaceous perennial plant of Hypericeae family widely distributed in Europe, Asia, and North Africa; it is also naturalized in North America. It is well known as a medicinal plant and its extracts are used as a healing and an anti-inflammatory agent (Di Carlo et al., 2001) since Ancient Greece. Nowadays, *H. perforatum* is used for the treatment of mild to moderately severe depression. The increasing attention on St. John's wort relies on the efficacy and the safety profile as an antidepressant medicine, which has been demonstrated in numerous clinical trials challenging the conventional antidepressant drugs, such as tricyclic antidepressants and serotonin reuptake inhibitors (Greeson et al., 2001).

Hypericum perforatum extracts contain a lot of constituents with documented biological activity including phenolic acids, a broad range of flavonoids, naphthodianthrones and phloroglucinols. The antidepressant activity of *H. perforatum* was first attributed to the naphthodianthrones hypericin, pseudohypericin, protohypericin and protopseudohypericin (Meruelo et al., 1988). Recent studies

Abbreviations: LC, liquid chromatography; NMR, nuclear magnetic resonance; SPE, solid-phase extraction; MS, mass spectrometry; ESI, electrospray ionization; DAD, diode array detector.

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revealed that the phloroglucinol hyperforin and its derivative adhyperforin, inhibit various neurotransmitter receptors (Laakmann et al., 1998). Additionally, flavonoids present in St. John's wort extracts have been shown to have antidepressive activities (Butterweck et al., 2000). Naphthodianthrones have also been discovered to act as antiviral agent and to inhibit the growth of a variety of neoplasmatic cell types (Kraus et al., 1996). Moreover, hyperforin has been shown to display antibacterial activity (Schempp et al., 1999) and was also proposed to act as a novel anticancer drug, due to its activity to inhibit apoptosis (Schempp et al., 2002).

Qualitative and quantitative variation in the content of secondary metabolites in *H. perforatum* is influenced by ecological and environmental effects, as well as physiological and genetic factors (Kosuth et al., 2003). Exposure of plant extract to light, converts protohypericin and protopseudohypericin into hypericin and pseudohypericin, respectively (Falk, 1999; Tolonen et al., 2003), and leads to the degradation of phloroglucinols, which are extremely sensitive to oxidation and unstable in solution when exposed to air (Tolonen et al., 2003). Since efficacy of medical preparations of *H. perforatum* is based on the whole mixture of metabolites (synergism), rather than the presence of a single constituent, the availability of methods allowing the analysis of the entire extract is a challenge.

The coupling of LC with spectroscopic techniques such as UV, MS or NMR provides a useful tool for rapid data collection and structure elucidation (Wolfender et al., 2003). LC/DAD is an effective technique for a rapid screening of mixtures, however, the light absorbance data obtained are insufficient for structure elucidation (Snyder et al., 1997). The use of hyphenated LC/UV/MS instrumentation has been reported in numerous applications (Wolfender et al., 1998). This technique is fast and the short time of exposure of the analytes to light and air limits their degradation. However, the MS and MS/MS data do not give detailed and conclusive structural information, especially when isomers of bioactive compounds are studied (Albert, 2004).

NMR spectroscopy is a powerful technique for structure elucidation of organic molecules. Therefore, the coupling of LC and NMR could lead to the complete assignment and structure determination of analytes. LC/NMR has become an important technique for the biomedical, pharmaceutical, environmental, food and natural products analysis, as well as for the identification of drug metabolites (Albert, 1999, 2004; Jaroszewski, 2005). However, whenever the concentration of analyte as eluted from LC column is not sufficient, the sensitivity of LC–NMR is a suspending factor for more sophisticated 2D NMR experiments (COSY, NOESY, HMQC). Recently, an alternative hyphenated technique, LC/SPE/NMR, has been applied to analyze Oregano plant extracts with very promising results (Exarchou et al., 2003). In this case, a SPE unit was inserted between the LC–UV unit and NMR spectrometer, in order to trap the eluting compounds onto SPE cartridges. Each one of the trapped compounds was eluted into the NMR probe with deuterated solvent. A limited number of studies on natural products (Seger et al., 2005; Miliauskas et al., 2005; Pulkaskas et al., 2005; Christoforidou et al., 2005; Clarkson et al., 2005; Exarchou et al., 2006) and drug metabolites (Godejohann et al., 2004) analysis using the on-line LC/SPE/NMR set-up are available in the literature, providing very interesting results.

A number of studies have been previously reported the analysis of St. John's wort extract constituents, based on LC/DAD/fluorescence (Li and Fitzloff, 2001) and LC/MS^2 (Brolis et al., 1998; Piperopoulos et al., 1997; Ganzera et al., 2002). Hansen et al. (1999) performed LC/NMR/MS measurements of major constituents of *H. perforatum*. However, the fractionation of the extract prior to its separation was necessary for the NMR detection. In the present work, we report the application of the combined use of LC/DAD/SPE/NMR and/or LC/UV/MS^2 to the identification of major compounds that are present in Greek *H. perforatum* species. In addition, the capabilities of LC/SPE/NMR set-up are investigated.

2. Results and discussion

The first step in LC/DAD/SPE/NMR analysis is to optimize the chromatographic separation and the SPE trapping conditions. Quercetin and rutin have been reported as constituents of *H. perforatum* extracts (Li and Fitzloff, 2001; Brolis et al., 1998; Hansen et al., 1999) and, thus, those flavonoids were used to test the trapping efficiency. In order to monitor the trapping process, a second UV/Vis detector was placed at the SPE cartridge outlet. Rutin and quercetin were fully trapped under the above experimental conditions. The cartridges with the trapped model compounds were dried and the flavonoids were eluted with deuterated acetonitrile into the NMR probehead, to reassure their trapping and transporting procedure. These trapping conditions were used to analyze the extract of St. John's wort.

In Fig. 1 the chromatographic profile at 270 nm of instrumental set-up, LC/DAD/SPE/NMR, of the extract from Greek *H. perforatum* is shown. The LC/UV trace revealed 10 major peaks. Peak **3** was trapped onto an SPE cartridge and after the drying step, it was eluted into the NMR flow probe and the ^{1}H NMR spectrum was acquired. The resulting signal-to-noise (S/N) ratio was not satisfactory, even after 2 h of acquisition. Our attempt to inject larger volumes in order to obtain a more informative spectrum was failed due to overloading phenomena. Thus, multiple trapping of the same analyte from repeated LC injections was used in order to increase their concentration in the NMR probehead. The obtained ^{1}H NMR spectrum after triple trapping showed a significant increase of the S/N ratio. Multiple trapping was used for all compounds marked in Fig. 1. Structure elucidation was based on a combination of UV, NMR and MS data.