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Capillary electrophoresis study on the base-catalyzed formation of bioactive oxidized metabolites of 20-hydroxyecdysone

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ABSTRACT

A novel capillary electrophoretic method was developed for the analysis and monitoring of the base-catalyzed autoxidation of 20-hydroxyecdysone, a worldwide used non-hormonal anabolic food supplement. An effective separation of the starting material and its bioactive oxidized derivatives was achieved by using sulfobutyl-β-cyclodextrin as selector at pH 11 and by fixing the separation voltage at +30 kV. Only a dilution step was inserted before injecting the sample, taken from the crude reaction mixture, to the capillary electrophoresis instrument. The same alkaline pH was used for the analysis as for the reaction, unlike the previously reported HPLC study where sample neutralization was required prior to the measurement. Due to the very short analysis time (6 min) in capillary electrophoresis, more frequent sampling and more detailed time scale analysis could be carried out. Furthermore, in contrast with the preceding HPLC results, the previously unobserved calonysterone could also be detected by capillary electrophoresis as a minor primary product. Our novel method demonstrated higher resolution than the one before. Baseline separation could be achieved and the resolution values were in the range of 1.9–7.0. The limit of detection was below 71 μg/ml, the relative standard deviation values of the migration time and peak area for intra- and inter-day precision were less than 10%. The more precise, direct monitoring of the time dependency of the oxidation process is expected to have a significant impact on yield optimization initiatives to allow related pharmacological studies in the near future.

1. Introduction

Ecdysteroids occur both as plant secondary metabolites (phytoecdysteroids) and insect molting hormones (zooecdysteroids). As secondary plant metabolites, they appear to have a complex role in plant-insect interactions [1], and in insects they regulate molting and metamorphosis, and promote growth of reproductive tissues, meiotic re-initiation, gametogenesis and embryogenesis [2]. Numerous, non-hormonal, and typically beneficial pharmacological effects of phytoecdysteroids have been reported in mammals including humans, such as effects on the function of various organs [3–6], on the protein, lipid and carbohydrate metabolism [7–10] and on the skin and oxidative stress [11–13]. The consequently expected health benefits and especially the anabolic effect led to the production and worldwide marketing of plant extracts with high ecdysteroid content as food supplements [14].

Concerning the oxidative metabolism of these compounds, 20-hydroxyecdysone (20E) is known to undergo autoxidation in presence of a strong base, which was first reported by Suksamrarn et al. in 1994 [15]. Recently, our research group reported an update to this reaction, also revealing that the formed oxidized derivatives exert significantly different and/or stronger bioactivities on the Akt phosphorylation in murine skeletal muscle cells as compared to their parental compound [16]. In particular, calonysterone and (5α)-9α,20-dihydroxyecdysone showed favourable activities over that of 20E on the aforementioned bioassay, suggesting the possibly more potent anabolic, antidiabetic, and cytoprotective activities of these metabolites as well.

The notable scientific and commercial interest on this class of natural products required the development of new methods for their detection and quantification. For such purposes, the use of various techniques has been described, including radio-immune

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assay (RIA), thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas chromatography (GC), as extensively reviewed over the last decades [17–20]. However, only a few publications are available on the use of capillary electrophoresis (CE) for the analysis of phytoecdysteroids. Micellar electrokinetic chromatography (MEKC) was used for the separation of 20E, 25-deoxy-11,20-dihydroxyecdysone, kaempferol 4′,7-dimethyl ether and biochanin A in the fruit, leaf and root of *Serratula strangulata Iljin* [21], and for the determination of 20E, ajugasterone C, polypodine B and ponasterone A [22]. In another study, capillary zone electrophoresis (CZE) was utilized for the determination of 20E, 3,7-dimethoxy-quercetin, acteoside and rutin in the herb and the flower of Lamium maculatum [23].

Capillary electrophoresis has become a reference separation technique because of the rapid analysis time and the efficiency that it provides [24]. Based on previous papers comparing CE and HPLC methods for the analysis of plant secondary metabolites [25–27], several advantages of CE can be highlighted. It demonstrates ultra-high separation efficiency, the separation media can easily be changed, and extremely small volumes of both sample and media are needed. In addition to these, it is commonly known that, prior to HPLC analysis, samples frequently require laborious pre-treatment e.g. solid-phase extraction, and the separation conditions are typically restricted to acidic-neutral pH due to the base-sensitivity of commonly used reversed-phase columns. In case of CE, however, only minor sample pre-purification is required when the analyte represents a high-enough concentration for identification, and a wide range of pH including highly alkaline is well tolerated by the instrument [28].

Accordingly, the aim of the present study was to develop a CE method for the separation of 20E and its bioactive oxidized metabolites forming in aerobic conditions upon base catalysis, and to utilize this method for directly monitoring the time dependency of the oxidation process.

2. Materials and methods

2.1. Materials

The starting material 20E isolated from the roots of *Cyanotis arachnoidea* with a purity of 90% was purchased from Shaanxi KingSci Biotechnology Co., Ltd. (Shanghai, China).

All the chemicals used for the synthetic reaction and the purification steps (NaOH, methanol and ethyl acetate) were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol used for HPLC analysis was of chromatographic grade and was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Phosphoric acid used as background electrolyte buffer (BGE) and sodium dodecyl sulphate were bought from Merck GmbH (Darmstadt, Germany).

Cyclodextrin derivatives: sulfobutyl ether β-cyclodextrin selector (SBEBCD; degree of substitution (DS) = 4), carboxymethyl-β-CD (CMBCD; DS = 3.5) and sulfobutyl-ether-γ-CD (SBEGCD; DS = 4) were purchased from CycloLab Ltd. (Budapest, Hungary).

2.2. Preparation of oxidized derivatives of 20E

Prior to use, the starting material 20E was re-crystallized from ethyl acetate – methanol (2:1, v/v) and thus reached a purity of 97.8% by means of HPLC-DAD. Base-catalyzed autoxidation of 20E was performed as previously published by our research group [16], with the minor difference that the final pH of the mixture was set to slightly acidic (pH ≈ 6). Briefly, 1 g of 20E was stirred at room temperature in 10% aqueous methanol containing 10 mg/ml of NaOH for 14 h followed by a pH adjustment of the final mixture. The final reaction mixture was fractionated through classical reverse-phase column chromatography with a stepwise gradient of methanol – water (3:7–6:4, v/v) resulting in 15 combined fractions. Fraction 2 was further purified through a CombiFlash® Rf + (Teledyne Isco, Lincoln, USA) apparatus with a 12 g HP Silica RediSep® Rf Gold column (Teledyne Isco, Lincoln, USA) with the use of *n*-hexane as mobile phase A and acetone as mobile phase B, the flow rate was 30 ml/min and the gradient was 0–20% B in 40 min leading to the isolation of compound **2** (78.2 mg). Fraction 8 was subjected to centrifugal partition chromatography (Armen Spot CPC 250 ml, Armen Instrument, Saint Ave, France), used in ascending mode with a solvent system of ethyl acetate – methanol – water (20:20:1, v/v/v), leading to the isolation of compound **5** (40.58 mg). Fraction 9 was crystallized from ethyl acetate – methanol (2:1, v/v), and compound **4** was obtained (115.06 mg).

Identification of the isolated compounds was achieved by comparison with fully characterized reference substances prepared during our previous study [16]. For this purpose, normal-phase (NP) TLC and reversed-phase (RP) HPLC was used. TLC was performed on silica plates (Silica gel 60F254, E. Merck, Germany), with a solvent system composed of ethyl acetate – ethanol – water (12:1:0.5, v/v/v) and visualizing the plates under UV light $\lambda_1 = 254$ nm, and under $\lambda_2 = 365$ nm after spraying them with vanillin/sulfuric acid reagent. HPLC-DAD (Jasco Co., Tokyo, Japan) was performed on a Kinetex XB C-18 column (5 μm, 250 × 4.6 mm) with an isocratic solvent system of 55% aqueous methanol at a flow rate of 0.8 ml/min.

2.3. Capillary electrophoresis

2.3.1. Capillary electrophoresis setup

Capillary electrophoresis was performed with an Agilent Capillary Electrophoresis ^{3D}CE system applying bare fused silica capillary of 64.5 cm total and 56 cm effective length with 50 μm I.D. (Agilent Technologies, Santa Clara, CA, USA). On-line UV absorption was monitored in the range of 200–600 nm by DAD UV–vis detector. The ChemStation software ver. B.04.01 (Agilent Technologies, USA) was used for data acquisition and handling.

The temperature was set to 25 °C. The samples were injected by 5×10^3 Pa pressure for 6s. The separation voltage was +30 kV. The detection with UV was at the maximum absorption wavelength (λ_{max}) of each compound (222, 228, 228, 247, 260 and 360 nm for compounds **4**, **2**, **1**, 20E, **3** and **5**, respectively). Between measurements, the capillary was rinsed subsequently by 0.1 M HCl, 1 M NaOH, 0.1 M NaOH and distilled water for 2 min, then with BGE for 3 min. Phosphoric acid buffer (10 mM) at pH 11 was applied as background electrolyte completed with 5 mM SBEBCD and the pH was adjusted by NaOH.

2.3.2. Evaluation of the method applicability

Linearity of the method for the evaluation of the concentration of 20E and its oxidized derivatives was evaluated by standards at least four concentration levels ranging between 25 and 1200 μg/ml with triplicate injections for each concentration. The whole calibration curves were made by plotting the integrated peak areas under the maximal wavelength of the targeted compound (y) against the corresponding concentration of the injected solutions (x μg/ml). Linear regression was performed using OriginPro 9.1 software (OriginLab Corporation, Northampton, Massachusetts).

Limits of detection (LOD) and limits of quantification (LOQ) were calculated for each material and were defined as $\text{LOD} = (b + 3s_{x/y})/a$ and $\text{LOQ} = (b + 10s_{x/y})/a$, where a, b and $s_{x/y}$ are the slope, the intercept and the residual standard deviation estimated from the linear regression, respectively [29].

Intra-day precision of the method was studied by triplicate injections of 1200 μg/ml concentration of 20E, compounds **3** and **5** and of 600 μg/ml concentration of compounds **2** and **4**. The