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Chiral separation of 2-hydroxyglutaric acid on cinchonan carbamate based weak chiral anion exchangers by high-performance liquid chromatography

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ABSTRACT

D- and L-2-Hydroxyglutaric acid (D- and L-2-HG, respectively) are metabolites related to some diseases (2-hydroxyglutaric aciduria, cancer), which make their identification and analysis crucially important for diagnostic purposes. Chiral stationary phases (CSP) based on *tert*-butylcarbamoyl-quinine and −quinidine (Chiralpak QN-AX and QD-AX), and the corresponding zwitterionic derivatives (Chiralpak ZWIX(+) and Chiralpak ZWIX(−)) were employed in a weak anion-exchange mechanism to perform the enantiomer separation of D- and L-2-HG without derivatization.

QD-AX CSP showed the most promising separation and therefore optimization of eluent, additives, and temperature, required for the baseline separation of solutes was carried out. Depending on experimental conditions resolution values ranged up to 2.0 with run times <20 min and MS-compatible conditions. Inversion on the elution order of D- and L-2-HG was possible by using the pseudo-enantiomeric QN-AX CSP.

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1. Introduction

2-Hydroxyglutaric acid is a clinically relevant chiral metabolite existing in two enantiomeric forms, i.e. D-2-HG and L-2-HG (Fig. 1a). Generally, their concentrations in humans are relatively low, with intracellular levels in normal cells below 0.1 mM [1]. However, high concentrations of these compounds have been identified in some patients, who suffer of particular diseases.

The best known of these diseases are D-2-hydroxyglutaric aciduria and L-2-hydroxyglutaric aciduria, which are reported for the first time in 1977 [2,3] and 1980 [4], respectively. Since then, multiple reports of both types of diseases have been published [5–18]. Also, cases of combined D,L-2-hydroxyglutaric aciduria have been reported [19].

The clinical phenotype of patients with D-2-hydroxyglutaric aciduria includes epilepsy, hypotonia and psychomotor retardation [11]. The clinical phenotype of patients with L-2-hydroxyglutaric aciduria is characterized mainly by developmental delay, epilepsy and cerebellar ataxia [11].

More recently, high concentrations of D-2-HG have been identified in cancer cells, including gliomas, glioblastomas and acute myelogenous leukemia (AML), with mutations of the enzymes isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) [13,20].

Mutated IDH enzymes reduce the compound 2-oxoglutarate (2-OG) to D-2-HG, considered an oncometabolite (or cancer-causing metabolite). 2-OG is a tricarboxylic acid (TCA) cycle intermediate and an essential cofactor for many enzymes [1]. The levels of D-2-HG in IDH mutant tumors can be extremely elevated, ranging from 1 mM to as high as 30 mM, hence being 10–300 times higher than the normal values [1].

Due to the clinical importance of D-2-HG and L-2-HG, different methodologies have been developed in order to identify and quantify their concentration in biological fluids enantioselectively. Indirect gas chromatographic enantiomer analysis using precolumn derivatization with 2-(−)-butanol as chiral derivatizing agent (CDA) for esterification and *O*-acetylation with acetic anhydride [3,4,8] or with (*S*)-(+)-3-methyl-2-butanol for carboxyl group derivatization and *O*-trifluoroacetylation [18] have been proposed. Some reports described direct GC enantiomer separation methods with achiral derivatization, e.g. with ethyl chloroformate, and subsequent analysis on cyclodextrin-based enantioselective columns [16,21]. A number of studies also reported indirect LC-enantiomer separation of 2-HG, e.g. after derivatization with diacetyl-L-tartaric anhydride [15], or with *N*-(*p*-toluenesulfonyl)-L-phenylalanyl chloride [22]. Only a few studies used direct liquid chromatographic

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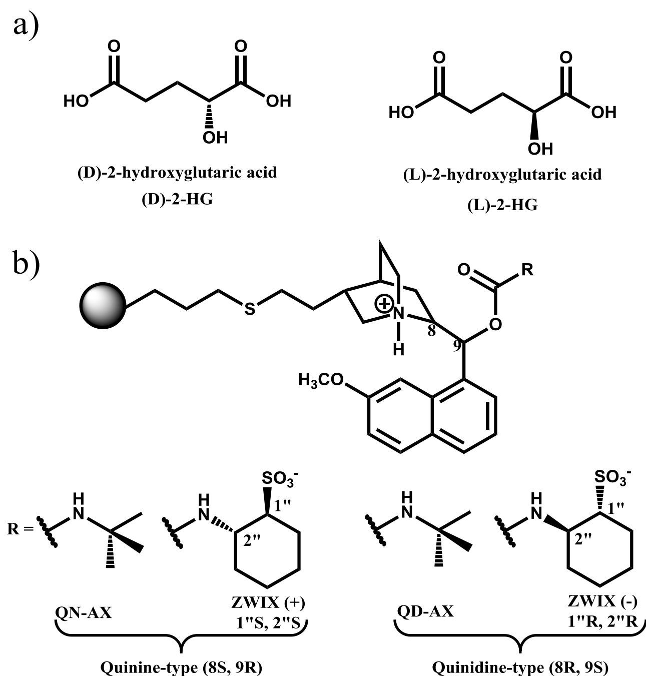

Fig. 1. (a) Enantiomers of 2-HG-, D-2-hydroxyglutaric acid and L-2-hydroxyglutaric acid, (b) chiral stationary phases tested in this study: Chiralpak QN-AX and QD-AX as well as Chiralpak ZWIX(+) and ZWIX(−).

enantiomer separation. In one study, D-2-hydroxyglutarate dehydrogenase activity was measured in cell homogenates derived from D-2-hydroxyglutaric aciduria patients using enantioselective ligand-exchange LC with D-penicilamine-based CSP and 2 mM Cu(II) acetate in water-MeOH (90:10; v/v) as eluent [23]. This method showed excellent enantioselectivity, but the Cu(II) ions in the eluent prevent its hyphenation to MS. In another study, enantiomeric separation of D- and L-2-HG was achieved by HPLC–MS using a ristocetin A glycopeptide antibiotic silica gel bonded column [17]. Last but not least, the enantioselective determination of D-2-HG in urine samples by using enantioselective membrane electrodes based on vancomycin and teicoplanin is worth mentioning [14].

In this study, a screening of the capability of four different chiral stationary phases (Fig. 1b) for the separation of the enantiomers D- and L-2-HG is performed. Conditions for the separation without derivatization are optimized by using HPLC–MS compatible conditions. This allows a straightforward translation of the procedure obtained in this work to a MS related platform in a subsequent step for the analysis of biologically relevant samples such as from cancer patients using a HPLC–MS/MS approach.

2. Experimental

2.1. Materials

All solvents used were of HPLC grade. Acetonitrile (ACN) was purchased from Panreac (Barcelona, Spain), methanol (MeOH) was obtained from VWR (Vienna, Austria), ethanol (EtOH) was obtained from Sigma–Aldrich (Munich, Germany) and 1-propanol (PrOH) from Merck (Darmstadt, Germany). The employed water was purified by a water filtration system from Elga Veolia (Paris, France). Formic acid (FA) was supplied by Roth (Karlsruhe, Germany). Acetic acid (AcOH), ammonia in methanol (NH_3), diethylamine (DEA) and triethylamine (TEA) were obtained from Sigma–Aldrich (Munich, Germany).

The racemic mixture D,L-2-hydroxyglutaric acid sodium salt and the enantiomerically pure compound L-2-hydroxyglutaric acid sodium salt were purchased from Sigma–Aldrich. The enantiomer elution order was assessed by injection of single enantiomer and/or non-racemic mixtures. The analytes were dissolved in water or methanol at approximate concentrations of 1.0 mg/mL.

2.2. Instrumentation and chromatographic method

All chromatographic measurements were performed on a 1100 Series HPLC from Agilent Technologies (Waldbronn, Germany) consisting of a solvent degasser, a binary pump, an autosampler, a column thermostat and a UV–vis detector. The HPLC system was connected to a Corona® Charged Aerosol Detector, CAD® from ESA Biosciences Inc., (Chelmsford, U.S.A.). The nitrogen flow of the CAD was adjusted to 35 psi. Data acquisition and analysis were done with ChemStation chromatographic data software from Agilent Technologies. The void volumes of the columns were determined by injecting a solution of 10% acetone in MeOH with detection at 280 nm.

Four different chiral stationary phases were employed in this study: a Chiralpak ZWIX(+) column (150 × 4 mm ID, 3 μm particle size), a Chiralpak ZWIX(−) column (150 × 4 mm ID, 3 μm particle size), a Chiralpak QD-AX (150 × 4 mm, 5 μm) and a Chiralpak QN-AX (150 × 4 mm ID, 5 μm particle size) from Chiral Technologies Europe (Illkirch, France).

The columns were conditioned with the selected mobile phase at a flow rate of 1.0 mL/min for at least 30 min, before performing analysis. Unless otherwise stated, the column temperature was kept at 25 °C.

3. Results and discussion

3.1. Column screening and elution order

Chiralpak ZWIX(+) and ZWIX(−) as well as Chiralpak QD-AX and QN-AX correspond to chiral zwitterionic ion-exchangers and chiral anion exchangers, respectively, based on carbamoylated cinchona alkaloid derivatives as illustrated in Fig. 1b. In the case of the chiral zwitterionic (ZWIX) selectors, successful separation of chiral acids [24], chiral bases [25,26] and zwitterionic chiral species [24,27,28] have been reported. Chiral weak anion-exchange (WAX) selectors have been employed for the chiral separation of amino acids derivatives [29–31], acids [32] and peptides [33]. Although many chiral acidic compounds have been successfully separated using both types of selectors, it should be pointed out that in most of these cases, the acidic compounds corresponded to structures in which not only the acid motif but also other functional groups were present as supportive interaction sites with the chiral selectors (SO). This includes in particular aromatic rings for π-π-interaction and amide or carbamate moieties with hydrogen donor-acceptor groups for H-bonding or dipole-dipole stacking. The lack of such interaction sites with strong directional nature makes the separation of enantiomers in the case of simpler molecules, like aliphatic hydroxy alkanoic acids, more difficult. The hydroxyl group can freely rotate so that the H-donor is less directed. Hydroxyl groups are furthermore strongly solvated in polar solvents such as water, which renders them less available for interaction with the chiral selector. In spite of these difficulties, lactic acid enantiomer separation has been reported by using QN-AX and QD-AX columns [34] and some 3-hydroxycarboxylic acids have been separated by using ZWIX(+) and ZWIX(−) columns using a polar organic elution mode [35]. Different ratios of ACN-MeOH were used in those cases for the elution of analytes, and normally formic acid or acetic acid as additives (the dissociation products of which represent coun-

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