Talanta 121 (2014) 178–186

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Ultra-fast separation of estrogen steroids using subcritical fluid chromatography on sub-2-micron particles

Lucie Nováková *, Petr Chocholouš, Petr Solich

Department of Analytical Chemistry, Faculty of Pharmacy, Charles University in Prague, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 12 September 2013
Received in revised form
19 December 2013
Accepted 24 December 2013
Available online 7 January 2014

Keywords:
Steroids
Estrogens
Subcritical fluid chromatography
Sub-2-micron particles
Method development

ABSTRACT

Estrogen steroids, represented by estradiol and its related substances, include both structurally very close and simultaneously different analogs. Their separation still remains an analytical challenge. Subcritical fluid chromatography (SbFC) on sub-2-micron particles was found to be an appropriate tool to obtain fast and efficient separation of nine target analytes. Among the four tested stationary phases charged hybrid modified with PFP (pentafluorophenyl) moiety was found to be the most convenient providing the fastest separation within 1.6 min using quick gradient elution with carbon dioxide and methanol as an organic modifier. However, complete separation was obtained also on other tested phases including bare hybrid stationary phase, hybrid stationary phase modified with 2-EP (2-ethylpyridine) and also C18, which is less typical in SbFC. The baseline separation on the latter columns was achieved by means of a temperature increase, a change in organic modifier type and gradient time increase respectively.

Quantitative performance was evaluated at optimized conditions and method validation was accomplished. Excellent repeatability of both retention times (RSD < 0.15%) and peak areas (RSD < 1%) was observed. The method was linear in the range of 1.0–1000.0 μg/ml for all steroids with the lowest calibration point being an LOQ, except for Δ-derivatives, that provided better sensitivity and thus LOQ of 0.5 μg/ml. The sensitivity was sufficient for the analysis of real samples although it was still five times lower compared to UHPLC-UV experiments.

1. Introduction

Supercritical fluid chromatography (SFC) is a separation technique widely used in pharmaceutical industry for chiral [1–3] and achiral separations [4–7] in both analytical and preparative scales. Supercritical conditions are reached when the substance occurs above its critical temperature and pressure. Such a mobile phase demonstrates lower viscosity and higher diffusivity relative to liquids. Therefore, minimum height equivalent to a theoretical plate values about similar to LC are obtained at linear velocities three to five-times greater than in LC [8]. Low critical values of carbon dioxide (temperature 31.1 °C and pressure 73.8 bar) allowed it to become the most preferred mobile phase in SFC. Due to its lipophicility, carbon dioxide enables elution of only a limited number of species. For the analysis of less lipophilic compounds the addition of an organic modifier is necessary in order to increase solvating power of carbon dioxide. It is necessary to emphasize, that the addition of an organic modifier dramatically increases the critical values of carbon dioxide. Therefore, under commonly used chromatographic conditions at about 120–150 bar and about 40 °C the mobile phase does not exist anymore in supercritical state, when higher percentage of organic modifier is added [9]. Therefore the technique would further be referred to as a subcritical fluid chromatography (SbFC), once the organic modifier was used, which has previously also been referred to as packed-column SFC.

SFC and SbFC have been already denoted as interesting methods for the separation of steroid compounds and were applied for the analysis of corticosteroids [10–12], estrogens [13,14], androstenone [15] and selected synthetic mixtures of steroids from various classes [16–18]. Hanson [19] and Berger et al. [20] evaluated retention behavior of steroids of different polarities and structures in SbFC using various stationary phases including bare silica and silica modified with phenyl, nitro, cyanopropyl, diol, C18, amino [19] and diol, cyanopropyl, sulfonic acid, C18 and phenyl respectively [20]. The importance of stationary and mobile phase polarity have also been discussed by Hanson besides temperature and pressure variations and their influence on selectivity using isocratic elution [21].

However, most of the works date back to 80s–90s and the separation runs were still in the range of 8–10 min or much longer (> 20 min), which is not anymore the state of the art in these kinds of analyses. Apparently, capillary SFC methods provided even longer analysis time (30–40 min) due to low solvating power

* Corresponding author. Tel.: +420 495 067 381.
E-mail address: nol@email.cz (L. Nováková).

0039-9140/$ - see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.12.056

of CO_2 mobile phases [18]. Only some selected androgen steroids were successfully separated in 3 min [22] or in less than 1 min analysis [6] using ultra-fast SbFC on sub-2-micron particles. A group of estrogen steroids that include both structurally very close and different analogs simultaneously, i.e. estradiol and its related substances, which should be monitored during QC (quality control) of the drug product according to the requirements of the drug master file (Fig. 1), represents an analytical challenge. They have been analyzed previously using LC-UV with only a partial success, as the separation of the main critical pair of estrone and Δ-estrone has not been reached even when using more than 20 min gradient analysis on different stationary phases [23]. Other approaches common for the analysis of estrogen steroids include LC or GC coupled to tandem mass spectrometry (MS/MS) [24–27]. When MS/MS detection is applied, the baseline separation of individual components is not critical except for the isomeric species, such as e.g. α- and β-estradiol, which still remains fairly challenging. Therefore, even these relatively modern LC or GC–MS/MS approaches often required substantially long analysis times (usually 15–20 min) [28]. The introduction of UHPLC enabled to shorten the analysis times bellow 10 min [29,30].

Recently, new SFC platforms and new stationary phases have been commercially introduced in order to extend application potential and reliability of SFC and SbFC methods. Similarly to UHPLC, sub-2-μm particles are of great importance, as they enable highly efficient and ultra-fast separations [6,22]. The aim of this work was to develop ultra-fast SbFC method for the separation of the group of structurally similar/different steroids. Individual parameters that influenced the separation and selectivity in the SbFC method are pointed out. The amount of contribution of these parameters to the change in method selectivity is discussed in detail. Quantitative aspects have still remained challenging in SFC and SbFC applications due to low repeatability of both retention times and injection process, which was critical especially with the old type of SFC instruments. The method repeatability, validation and the applicability to real sample is also shown.

2. Experimental

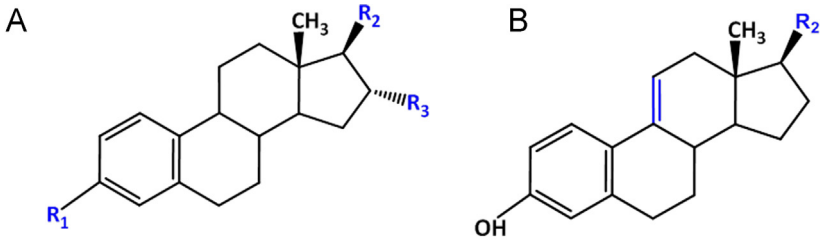
2.1. Chemicals and reagents

Reference standards of steroids were used for the purpose of this study. Estriol (99.9%), β-estradiol (>98%), estrone (>99%) and ethinylestradiol (≥98%) were obtained from Sigma-Aldrich, Czech Republic. α-estradiol hemihydrate (98.8%), estradiol-3-methylether (98.3%), and estradiol-17-acetate (99.3%) were obtained from Vetranal, Germany. 1,3,5,(10),9(11)-estratetraen-3, 17-β-diol designated as Δ-estradiol and 1,3,5,(10),9(11)-estratetraen-3-ol, 17-on designated as Δ-estrone were obtained from Steraloids, USA. Methanol, ethanol, propan-2-ol, acetonitrile gradient grade and tetrahydrofuran HPLC grade were provided by Sigma-Aldrich.

2.2. Ultra high performance subcritical fluid chromatography

The supercritical fluid chromatography system Acquity UPC^2 (Waters, Milford, USA) consisted of Acquity UPC^2 binary solvent manager, Acquity UPC^2-FL sample manager, Acquity UPC^2 convergence manager, Acquity column manager and Acquity UPC^2 PDA detector.

All injected solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill mode was set up to inject 1 μL. Methanol was used as a needle wash solvent. The separation was performed using four SFC dedicated stationary phases including: Acquity UPC^2 BEH, Acquity UPC^2 BEH 2-EP, Acquity UPC^2 CSH PFP and Acquity UPC^2 HSS C18 SB, all of them at 100 × 3.0 mm and 1.7 μm particles. Gradient elution was performed using CO_2 (>99.995%, LindeGas, Czech Republic) and various modifiers including methanol, ethanol, propan-2-ol and acetonitrile at flow-rate 2.5 ml/min. The temperature was optimized in the range of 40–90 °C. The BPR (back-pressure regulator) pressure was typically set-up to 2000 psi and the variations were observed in the range of 1500–2500 psi. UV detection was performed at 225 nm.



	compound		R1	R2	R3	Log *P*	pK$_a$
1	methoxy-estradiol	EST-me	-OCH$_3$	-OH		5.03	15.07
2	estradiol 17-acetate	EST-ac	-OH	-O-CO-CH$_3$		4.98	10.26
3	estrone	ESN	-OH	=O		4.40	10.25
5	α-estradiol	α-EST	-OH	··OH		3.91	10.27
6	ethinylestradiol	et-EST	-OH	-OH, -CΞCH		4.00	10.24
7	β-estradiol	β-EST	-OH	-OH		3.91	10.27
9	estriol	ESTRI	-OH	-OH	-OH	2.91	10.25
4	Δ-estrone	d-ESN		=O		3.62	10.41
8	Δ-estradiol	d-EST		-OH		3.59	10.39

Fig. 1. The structures of estrogen steroids included in this study and their physico-chemical properties. Most of the steroids are derived from structure (A), except for Δ-estradiol and Δ-estrone, that are derived from structure (B).