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Greener bioanalytical approach for LC/MS–MS assay of enalapril and enalaprilat in human plasma with total replacement of acetonitrile throughout all analytical stages☆

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ABSTRACT

Green bioanalytical approaches are oriented toward minimization or elimination of hazardous chemicals associated to bioanalytical applications. LC/MS–MS assay of enalapril and enalaprilat in human plasma was achieved by elimination of acetonitrile from both sample preparation and chromatographic separation stages. Protein precipitation (PP) by acetonitrile addition was replaced by liquid–liquid extraction (LLE) in 1-octanol followed by direct large volume injection of the organic layer in the chromatographic column operated under reversed phase (RP) separation mechanism. At the mean time, acetonitrile used as organic modifier in the mobile phase was successfully replaced by a mixture of propylene carbonate/ethanol (7/3, v/v). Three analytical alternatives ((I) acetonitrile PP + acetonitrile based chromatographic elution; (II) 1-octanol LLE + acetonitrile based chromatographic elution; (III) 1-octanol LLE + propylene carbonate/ethanol based chromatographic elution) were validated and the quality characteristics were compared. Comparison between these alternative analytical approaches was also based on results obtained on incurred samples taken during a bioequivalence study, through application of the Bland–Altman procedure.

1. Introduction

The introduction of the concept of green analytical chemistry dates from the late nineties [1,2]. The definition of this concept was comprehensively formulated by Keith [3,4]: "the use of analytical chemistry techniques and methodologies that reduce or eliminate solvents, reagents, preservatives and other chemicals that are hazardous to human health or the environment and that may also enable faster and more energy-efficient analyses without compromising performance criteria". More often the most difficult task is to translate from existing methods to greener ones keeping unaltered the performance criteria. Topics on greening of analytical chemistry were extensively discussed and reviewed [5–9]. Aspects focusing specifically on chromatographic methods are also available [10–12]. A discussion about green bioanalytical principles was recently published [13]. Implementation of the green chemistry principles in sample preparation conducted to the following development directions [14]: miniaturization of liquid–liquid extraction [15,16], elimination or minimization of solvent use [17], increasing the role of solid phase extraction in sample preparation [18,19]; increased use of membrane supported liquid phase (micro)extraction [20,21]; use of ionic liquids as extraction media [22] or ionization media from the solid state [23,24]. In liquid chromatography, two main directions for greener alternatives are currently explored. The first one deals with reduction of solvents and additives through reducing columns internal diameters (from analytical to narrow or micro bore ranges) and dimensions of packings (from 5 to 3 and sub-2 μm particle sizes) [25,26]. The second one refers to replacement of acetonitrile and/or methanol in the mobile phases with less harmful and environmental friendly alternatives such as water [27], ethanol (EtOH) or iso propanol [28], propylene carbonate (PC) [29] and carbon dioxide (either in sub-critical or supercritical state) [30–32].

We actually tried to turn a somewhat "classical" bioanalytical method in a greener one. The first step consisted in the replacement of acetonitrile (ACN) during the sample preparation stage (protein precipitation). Liquid–liquid extraction in 1-octanol was considered as a possible alternative. Direct large volume injection from the organic layer is thus possible. The fundamentals referring to large volume injection of diluents non-miscible with the mobile

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phase were already highlighted [33,34]. Bioanalytical applications based on LLE in 1-octanol are also available and were successfully used in bioequivalence studies [35,36]. The following step aimed to eliminate ACN from the mobile phase. Propylene carbonate (PC)/methanol mixtures have been already prospectively used as an alternative to ACN in mobile phases designated for RPLC applications [37–39]. Thus, the possibility of using PC/EtOH instead of ACN was investigated.

The couple of enalapril (relatively inactive prodrug)/enalaprilat (active metabolite) in plasma matrix seemed suitable for the application of the "greening" concept for the following reasons: (1) significant difference regarding the hydrophobic characteristics ($\log P$ is −1.05 for enalaprilat and 0.59 for enalapril); (2) the application might be taken as a "worst case" if considering liquid extraction ability of target compounds in a hydrophobic environment as octanol ($\log P = 3$); (3) suitable retention of enalaprilat requires water rich mobile phases; (4) elution of enalapril as a symmetric peak usually needs temperature and richer organic solvent containing mobile phases, to avoid selectivity against the *cis* and *trans* rotamers; (5) pharmacokinetic characteristics of target compounds are well known; however, for obtaining accurate plasma concentration vs. time profiles, sensitivities below 1 ng/mL level are required; (6) sufficient experimental data about the bioassay of the target analytes in plasma is available. In such a context, translation from "classic" to "green" analytical solutions without any compromise in terms of method performance should not be necessarily considered an easy task.

Sample preparation approaches used during bioanalytical methods for assaying enalapril and enalaprilat in plasma matrices are the following: one step protein precipitation (PP) by acetonitrile [40] or methanol [41] addition; solid-phase extraction [42–45]; liquid–liquid extraction [46]. The lowest quantitation limits (LLOQ) reported in literature that were obtained with these methods were situated at the 0.1 ng/mL level for both compounds [45,46].The "classic" approach (Method 1 using PP with ACN and ACN based chromatographic elution) was gradually modified to Method 2 (LLE in 1-octanol and ACN based chromatographic elution) and finally to a final stage (Method 3 using LLE in 1-octanol and PC/EtOH based chromatographic elution). The three alternatives were validated and their quality characteristics were compared. For a better insight, incurred samples resulting from a bioequivalence study (2 volunteers, after administration of reference and tested products) were successively analyzed by means of the three approaches and the results were compared based on the Bland–Altman methodology.

2. Experimental

2.1. Reagents

Acetonitrile HPLC gradient grade from Merck (Darmstadt, Germany), ethanol and propylene carbonate CHROMASOLV® grade from Sigma–Aldrich (Taufkirchen, Germany) were used during experiments. Water for chromatography (resistivity of minimum 18.2 MΩ and TOC of maximum 30 ng/mL) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument. Enalapril maleate ((2*S*)-1-[(2*S*)-2-{[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl]pyrrolidine-2-carboxylic acid, cat. no. 1235300) and enalaprilat (2*S*)-1-[(2*S*)-2-{[(1*S*)-1-carboxy-3-phenylpropyl]amino}propanoyl]pyrrolidine-2-carboxylic acid, cat. no. 1235274 were reference standards from U.S. Pharmacopeia (Twinbrook Parkway, Rockville, USA). The two internal standards were 4-[2-(5-chloro-2-methoxybenzamido)ethyl]benzene-sulphonamide or glibenclamide impurity A (IS1) and 4-[2-(5-methylpyrazine-2-carboxamido)ethyl]benzene sulphonamide or glipizide impurity A (IS2) certified reference substances from European Pharmacopoeia (Strasbourg, France). 1-Octanol and formic acid were extra pure grade from Merck.

2.2. Equipments

Experiments were performed with an Agilent 1200 SL series LC/MSD (Agilent Technologies) system consisting of the following modules: degasser (G1379B), binary pump (G1312B), automated injector (G1367C and the corresponding thermostat G1330B, respectively), column thermostat (G1316B), ESI standard interface (G1948B), and triple quadrupole mass spectrometric detector (model 6410 QQQ). System control, data acquisition and interpretation were made with the Agilent MassHunter software version B 04.01 (B4114 Patch 1) incorporating both qualitative and quantitative packages. The Multi Reax vortex from Heidolph (Schwabach, Germany) and the thermostated centrifuge model Universal 320R from Hettich (Tuttlingen, Germany) were used.

2.3. Sample preparation

The two sample preparation alternatives being considered are:

A. Plasma protein precipitation with acetonitrile (PP with ACN): An aliquot of 0.4 mL from a solution containing 50 ng/mL IS1 in ACN was added to 0.2 mL plasma sample. Phases were vortexed for 10 min and then centrifuged at 25 °C and 9000 × *g* for 5 min. A volume of 5 μL from the supernatant was injected in the chromatographic column.
B. Liquid–liquid extraction in 1-octanol (LLE in octanol): A 20 μL aliquot of formic acid was added to the plasma sample (0.2 mL). The sample is vortexed for 5 min. Extraction is made in 0.3 mL of 1-octanol already containing the internal standard (50 ng/mL). The two phases were vortexed for 10 min and then centrifuged at 25 °C and 9000 × *g* for 5 min. The 1-octanol upper layer could be easily and quantitatively transferred in the injection vial, as the phases are well separated by a thin highly viscous film (probably of lipidic nature) aggregated at the interface between layers. A volume of 75 μL from the extractant is directly injected to the chromatographic column. Vials were thermostated in the autosampler at 25 °C.

2.4. Chromatographic methods

A Zorbax SB-C18 Rapid Resolution column, 50 mm length, 4.6 mm internal diameter and 1.8 μm particle size (Agilent Technologies, cat. no. 822975-902) fitted with a Phenomenex Guard Cartridge C18, 4 mm × 2 mm (prod. no. AJO-4286) were used and thermostated at 50 °C.

The first approach was based on ACN elution. The components of the mobile phase were 0.1% formic acid in ACN (Solvent A) and aqueous 0.1% formic acid solution (Solvent B). A gradient elution was applied, according to the following profile (time – min/Solvent A – %/flow rate – mL/min): 0.0/10/0.8 → 5.0/70/0.8 → 5.01/100/0.8 → 5.50/100/0.8 → 6.0/100/1.2 → 6.01/10/1.2 → 8.0/10/1.2. The composition profile between 5.0 min and 6.0 min is designated to the elimination of 1-octanol from the column. The period between 6.0 min and 8.0 min is dedicated to column re-equilibration (column re-equilibration at the flow rate of 0.8 mL/min is obtained during the period covering the consecutive injection). The stop time of the method is 8 min. The drawing/dispensing speed of the autosampler was set to 100 μL/min, to compensate for the high viscosity of 1-octanol. In the case of ACN elution, IS1 was used as internal standard.