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Two novel solvent system compositions for protected synthetic peptide purification by centrifugal partition chromatography

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ABSTRACT

Protected synthetic peptide intermediates are often hydrophobic and not soluble in most common solvents. They are thus difficult to purify by preparative reversed-phase high-performance liquid chromatography (RP-HPLC), usually used for industrial production. It is then challenging to develop alternative chromatographic purification processes. Support-free liquid–liquid chromatographic techniques, including both hydrostatic (centrifugal partition chromatography or CPC) and hydrodynamic (counter-current chromatography or CCC) devices, are mainly involved in phytochemical studies but have also been applied to synthetic peptide purification. In this framework, two new biphasic solvent system compositions covering a wide range of polarity were developed to overcome solubility problems mentioned above. The new systems composed of heptane/tetrahydrofuran/acetonitrile/dimethylsulfoxide/water and heptane/methyl-tetrahydrofuran/N-methylpyrrolidone/water were efficiently used for the CPC purification of a 39-mer protected exenatide (Byetta®) and a 8-mer protected peptide intermediate of bivalirudin (Angiox®) synthesis. Phase compositions of the different biphasic solvent systems were determined by ^{1}H nuclear magnetic resonance. Physico-chemical properties including viscosity, density and interfacial tension of these biphasic systems are also described.

1. Introduction

<SE>Please note that in some sentences words appear in different font and font size here and else where.<SE>Centrifugal partition chromatography (CPC) is a preparative support-free liquid-liquid chromatographic technique, based on the difference in partitioning behaviour of analytes between at least two immiscible liquid phases [1]. One liquid phase is kept stationary inside the column, thanks to a constant centrifugal force field, while the other one is pumped through it. As compared to solid support chromatographic techniques, CPC offers advantages mainly due to the large volume of the liquid stationary phase available in the column and the absence of chromatographic solid support. CPC is often characterised by a high capacity, a low solvent consumption and a total recovery of the loaded sample [2]. The biphasic liquid systems used in CPC are usually composed of a mixture of three or four solvents and/or solutions. The two phases of the same solvent system are in a thermodynamic equilibrium. The high diversity of available biphasic systems makes CPC a versatile tool for the separation of a wide variety of compounds such as natural products or synthetic pharmaceuticals [3,4]. Nevertheless, mobile and stationary phase development in CPC (or in CCC) remains sometimes complicated. Indeed, as the CPC column is filled with two liquid phases, selection of a two-phase solvent system is similar to selection of an HPLC column and its eluent. In CPC, the possibilities of solvent combinations to form a biphasic system are almost limitless. Thus, the development of a small set of useful phases to fit many applications continues to be a major challenge.

Once the solvent combination for which the sample is freely soluble has been found (the goal of CPC is preparative), it is important to adjust the different solvent proportions to obtain distribution coefficients of the target analytes between 0.5 and 2 (this ideal elution area called the “sweet-spot”) if the elution development mode is used [4–7].

The multi-solvent system approach [6] has proven to be a useful tool to find a system with a satisfactory distribution coefficient (K_D) and selectivity. The most popular scales of biphasic solvent systems are: a) the Arizona system (or HEMWat), composed of heptane (or

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hexane), ethyl acetate, methanol and water, b) the OKA scale composed of hexane, ethyl acetate, *n*-butanol, methanol and water and c) the acetone scale composed of heptane, toluene, acetone and water [6,8]. For a given CPC column geometry, the physical properties (viscosity, interfacial tension and density difference) of an ideal solvent system are those that allow the highest mass transfer of solutes from the stationary to the mobile phase (and *vice versa*) and the highest stationary phase retention in the CPC column. For example, a low viscosity generally improves mass transfer phenomena and thus the efficiency of the chromatographic system [9]. Mass transfer increase can also be attempted by increasing the mobile phase flow rate and the rotational speed [10], but the adjustment of these parameters is limited by a risk of exceeding the apparatus pressure limit or decreasing the stationary phase retention and thus the resolution [11].

Furthermore, the solvents have to be compatible with the application and should not react with or promote degradation of the solutes. Additional factors such as volatility, toxicity, environmental, regulatory and economic aspects, also require attention.

A variety of two-phase solvent systems used for free or protected peptides purification in the elution mode can be found in the literature [12–16]. Nevertheless, protected non-ionic synthetic peptides are often hydrophobic and not soluble in the most common solvent systems used for peptide purification. These peptides require thus the development of new biphasic solvent systems. Two peptides – a 39-mer protected exenatide [17] and a 8-mer protected peptide intermediate of the bivalirudin [18,19] synthesis – both well known to cause purification problems mainly due to their low solubility in common HPLC solvents – were used as models. Bivalirudin and exenatide were both approved by regulatory agencies (Food and Drug Administration, European Medicines Agency) and are respectively the active substances for Angiox® (anticoagulant drug) and Byetta® (treatment of diabetes mellitus type 2). The production of these two drugs involves a final purification by RP-HPLC. This technique is generally used in production today, although there are alternatives such as medium pressure chromatography, Craig apparatus, crystallization, etc. For this work, the purification of the two synthetic intermediates by CPC was achieved after developing two original biphasic solvent system families composed of heptane/THF/MeCN/DMSO/water and of heptane/MeTHF/NMP/water.

2. Experimental

2.1. Chemicals

Heptane, tetrahydrofuran (THF), 2-Methyltetrahydrofuran (MeTHF), acetonitrile (MeCN), N-Methyl-2-pyrrolidone (NMP) and trifluoroacetic acid (TFA) were purchased from Carlo Erba (Ronado, Italy). Dimethyl sulfoxide (DMSO) was purchased from VWR (Fontenay-sous-bois, France). Water was purified by de-ionization and reverse osmosis. The 39-mer protected exenatide (Boc-His(Trt)-Gly-Glu(OtBu)-Gly-Thr(tBu)-Phe-Thr(tBu)-Ser(tBu)-Asp(OtBu)-Leu-Ser(tBu)-Lys(Boc)-Gln(Trt)-Met-Glu(OtBu)-Glu(OtBu)-Glu(OtBu)-Ala-Val-Arg(Pbf)-Leu-Phe-Ile-Glu(OtBu)-Trp(Boc)-Leu-Lys(Boc)-Asn(Trt)-Gly-Gly-Pro-Ser(tBu)-Ser(tBu)-Gly-Ala-Pro-Pro-Pro-Ser(tBu)-NH_2) and the 8-mer protected peptide intermediate of bivalirudin synthesis (Glu(OBzl)-Glu(Obzl)-Ile-Pro-Glu(OBzl)-Glu(OBzl)-Tyr(Bzl)-Leu(OBzl)) were provided by Lonza (Visp, Switzerland). The batches used for this work came from production lines. The entire processes for the production of both bivalirudin and exenatide were approved by regulatory agencies (Food and Drug Administration, European Medicines Agency). Drug Master File numbers for US are: 12797 for Bivalirudin – Angiomax®, and 19646 for exenatide – Byetta®.

2.2. Preparation of the two-phase solvent systems

The biphasic systems (100 mL) were prepared by mixing the solvents in the suitable proportions in a separatory funnel. They were vigorously shaken at room temperature and then allowed to settle until the phases became limpid.

2.3. Settling time and density measurement

Settling time was measured using the procedure described by Ito [5]; 2 mL of each phase were delivered into a graduated cylinder of 5 mL capacity, which was then capped and gently inverted for five times to thoroughly mix the contents. The cylinder was then immediately placed in an upright position to measure the time required for the two phases to form clear layers with a distinct interface.

Densities of the upper and the lower phases of each biphasic system were calculated after measuring the mass of an equal volume of water and of each phase.

2.4. Viscosity measurement

Kinematic viscosities were measured at 22 °C using a Rheomat RM300 routine viscometer (Neurtek, Eibar, Spain).

2.5. Interfacial tension measurement

Interfacial tensions were measured at 22 °C using the rising drop method with a Tracker S automated drop tensiometer (Teclis, Longessaigne, France). After separation of the phases, a syringe was used to generate a drop of the lower phase in a 25 mL cuvette containing the upper phase. The control unit of the instrument oscillates the observed drop to determine complex elastic modulus and trace the interfacial tension.

2.6. Distribution coefficient measurement

Distribution coefficients (K_D) were evaluated by HPLC as follows: a small amount (about 2 mg) of the sample was added to the equilibrated solvent phases (1 mL each) in a test vial. The vial was vigorously shaken. After decantation, 250 μL of each phase was diluted with 250 μL of the fresh conjugate phase and 500 μL of methanol (to mix the two phases and obtain a single phase) for HPLC analysis. The K_D value was expressed as the ratio of the peak area of the target compound in the upper phase divided by the one obtained for the lower phase.

2.7. Phase composition determination by ^1H NMR

The exact composition of each liquid phase at each step of this procedure was determined by ^1H NMR. A 200 μL aliquot of each phase was mixed with 300 μL of chloroform-d for the upper phase and DMSO-d6 for the lower phase, respectively. Analyses were achieved on a Bruker Avance 500 spectrometer (^1H at 500.13 MHz) (Bruker, Germany). Spectra were acquired at 298 K with 1 scan (no dummy scan) over a spectral width of 12 ppm, using the standard zg pulse programme and a 20 s relaxation delay. The ^1H NMR signals of heptane tetrahydrofuran, acetonitrile, dimethylsulfoxide, water, methyl-tetrahydrofuran and N-methylpyrrolidone were integrated in all spectra and the volume percentage (V %) of each solvent was calculated as V % = (A/n) × (MW/d), with A = signal area, n = number of proton(s), MW = molecular weight of the solvent and d = density of the solvent at 298 K. Measurements were performed in triplicate.