Journal of Chromatography A, 1218 (2011) 6053–6060

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Gradient elution in counter-current chromatography: A new layout for an old path

Svetlana Ignatova[a,*], Neil Sumner[b], Nicola Colclough[b], Ian Sutherland[a]

[a] Brunel Institute for Bioengineering, Brunel University, Uxbridge UB8 3PH, UK
[b] CIRA Chemistry, Astra Zeneca UK Limited, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

ARTICLE INFO

Article history:
Available online 27 February 2011

Keywords:
Gradient elution
Counter-current chromatography
Model samples
Solvent systems
Method development

ABSTRACT

Gradient elution in CCC is a powerful tool, which needs further systematic development to become robust and easy to use. The first attempt to build a correlation between gradient elution profile and distribution ratio (K_D) values for model mixtures containing typical representatives of pharmaceutical compounds is presented in this paper. The three step estimation of the solvent system composition of a heptane–ethyl acetate–methanol–water (HEMWat) series is described. The estimation is based on simple measurements of initial and final stationary phase retention for gradient elution run, calculating gradient distribution ratio and correlating it with static K_D against HEMWat number.

1. Introduction

While counter-current chromatography (CCC) has frequently been exploited for the isolation of active ingredients from various natural products [1,2], very little research has been performed on the isolation of small synthetic molecules. Recent developments in high "g-field" centrifuges and scale-up have demonstrated that CCC can effectively compete with HPLC for preparative and pilot scale separations by targeting one or two components from a complex, crude mixture [3–5]. However, to make CCC more effective for drug discovery, further research on rapid method development and fast complex mixture separations is urgently needed.

A successful CCC separation relies on the choice of an appropriate immiscible solvent system. Compared to solid-support chromatography, the selection of CCC solvent systems is equivalent to simultaneously choosing both the solid column matrix and the mobile liquid phase. There are a variety of appropriate immiscible solvent systems available; one of the most commonly used is a mixture of heptane, ethyl acetate, methanol and water in different ratios, often referred to as the Arizona system developed by Margraff and Foucault [6] or the HEMWat system derived from it [7]. The suitability of a given solvent system is empirical and generally estimated using its key parameter – the partition coefficient (K_D) of the target compound(s) between the two phases [1]. In the case of a complex mixture, the whole selection process can be by trial-and-error, and can therefore be time consuming.

Gradient elution is one approach to overcome this challenge, but its application in liquid–liquid chromatography is not well understood as a gradient set up in the mobile phase can and does change the composition of the liquid stationary phase, so its application is not as straightforward as in HPLC. Common gradient systems transferred from HPLC to CCC include a temperature gradient, a stepwise flow gradient, and stepwise and linear gradients of mobile phase components [2]. However, most of these examples have been used for "one-off" natural product separations.

The first gradient elution in CCC used the reversed phase mode and was demonstrated on the separation of seven dipeptides by Ito and Bowman in 1973 [8]. The centrifuge was rotating at 750 rpm but had a low "g-level" and did not have any temperature control. The latter became important when instrument development led to creating high speed "J" type centrifuges (84 g) followed by high performance "J" type centrifuges rotating at up to 240 g as both types generate heat. However, this increase in temperature can have a positive effect as long as temperature is constant during separation (thermostated columns). The slightly elevated temperatures (25–35 °C) result in better solubility of crude samples and therefore, better partitioning and throughput. The traditionally used aqueous–organic solvent systems are quite stable within this temperature range. The volume ratio of two phases might change but the two-phase structure will remain. However, in the case of non-aqueous systems temperature control is an important factor. Lower temperature assures a two-phase system while higher temperature facilitates partitioning of target compounds and shortens the separation time. The compromise between lower and higher temperatures has been applied to the isolation of trans-lycopenes from tomato paste with the

* Corresponding author. Tel.: +44 1895 266911; fax: +44 1895 274608.
E-mail address: svetlana.ignatova@brunel.ac.uk (S. Ignatova).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.02.052

non-aqueous phase system hexane–dichloromethane–acetonitrile [9].

If the chosen solvent system provides a good range of K_D values (~0.2–5.0) and separation factors α (>1.2) for multiple targets, but the separation time is too long, it can be shorten by using a stepwise increase in flow rate. There are a number of publications describing such an approach [10–20]. The general idea is to start the separation at lower flow rate. This would give sample enough time to get diluted in the column and therefore, minimise the additional displacement of stationary phase after injection of a concentrated sample and let compounds with the small K_D values (<1.5–2) elute with good resolution. This is followed by an increase in flow rate which leads to faster elution of the remaining targets without loss of separation efficiency.

When the solvent system does not provide an adequate separation of all compounds from a complex mixture, the most effective way of improving the separation is to change the composition of mobile phase, in other words – apply a gradient. This can lead to compounds eluting faster by rapidly changing the polarity of the mobile phase. Almost 15 years after Ito's first publication [8] this approach was demonstrated on the separation of flavonol glycosides from Ginkgo biloba in normal phase gradient elution in CCC [21]. The authors used a 2-butanol exponential gradient in ethyl acetate–2-butanol–water solvent system. Later in 1995 a normal phase gradient was transferred to centrifugal partition chromatography (CPC), another type of liquid–liquid chromatography [6], for the separation of anthocyanins from grapes using ethyl acetate–1-butanol–acidic water [22]. Furthermore, in 1997 this methodology was successfully scaled up from a 240 ml to a 5 l CPC centrifuge [23]. Since then the butanol linear gradient has become very popular and it is being widely used [24]. When CCC instruments became available in China, it gave a real boost to CCC development and its application to Chinese natural product separations [2] including use of gradient elution. However, most of the work involved stepwise elution rather than linear gradient, which is mainly due to the ancillary equipment availability [25–34]. An interesting example of a 3 step butanol gradient in normal phase CCC (n-hexane–1-butanol–0.05 M NaOH) combined with a descending stepwise flow gradient was published by Du in 2004 for the isolation of pentacyclic triterpene aglycones and glycosides of the ursane type from a herbal extract [35]. Increasing butanol content in the mobile phase resulted in a change of the stationary phase composition and its volume. To keep stationary phase retention as high as possible the author gradually decreased flow rate from 4 to 1.5 ml/min, which provided satisfactory separation of target compounds.

Recently, the application of pH-gradient [36–38] and salting-out gradient [39] as a polarity change approach for isolation of charged molecules in CCC/CPC has been reported. The presence of pH-modifier or salt in the solvent system often improve separation efficiency as they increase solubility of crude material and depress emulsification typical for polar herbal extracts and therefore, stabilises stationary phase retention [20].

The complication of gradient elution in CCC is that any change in mobile phase composition will also lead to the change of stationary phase composition due to its liquid nature. Therefore, in the solvent systems suitable for gradient elution in CCC/CPC one of the phases should have reasonably stable composition while another one would undergo a vast change. This feature of gradient elution was described and studied by Conway [40] and Foucault [41,42]. The latter suggested use of ternary diagrams to predict stability of the stationary phase and even calculate a composition of initial and final phases for gradient elution. Authors used hexane–methanol–water and chloroform–methanol–water solvent systems as the most appropriate for gradient elution. Foucault et al. demonstrated a successful separation of amino acids and peptides with a hexane–1-butanol–water system [41]. Unfortunately, this work was not taken any further despite other attempts to model gradient in CCC/CPC [43,44]. Ternary solvent systems generally consist of two immiscible solvents to assure two phases and the third solvent is miscible with both and partitioning between them. Such systems have a good range of polarity but it is not always good enough for separation of closely related compounds. In this case, quaternary systems like heptane–ethyl acetate–methanol–water (HEMWat) can be useful because each phase contains a modifier, which is partly partitioning in the opposite phase. This was successfully demonstrated by Leitao's group in 2005 [45,46]. Methanol step gradient was used in separation of free and glycosylated flavonoids from Brazilian natural product. Moreover, the authors used a "fifth solvent" approach for further purification of one of the fractions [46]. Addition of butanol into HEMWat system allowed achieving the separation.

Gradient elution in CCC is a powerful tool, which needs further systematic development to become robust and easy to use. Applying a gradient will lead to an increase in selectivity as it covers a higher polarity range or will allow the separation of co-eluting compounds. Using solvent system providing better solubility for the crude material as a starting point and then moving to the solvent system where actual separation occurs can minimise or even eliminate solubility issues. Gradient elution can be a very elegant solution for improving the hydrodynamic stability of a solvent system. This is particularly important for the separation of lipophilic compounds using non-aqueous systems [47]. The authors started from an aqueous–organic system, which is more stable to retain, and then substituted water with acetonitrile ending with a non-aqueous system, which is less stable for retaining.

In this work, we have made the first attempt to build a correlation between the gradient elution profile and K_D values for a model mixture containing typical representatives of pharmaceutical compounds using an analytical CCC instrument. All previous research in this area was carried out on semi-preparative or preparative scale instruments. Changing the mobile phase composition results in increased or decreased elution times for the various target components in the mixture, due to a change in the polarity of the liquid phases. As a result, the separation time can vary depending on the choice of target. It will allow us to create a template for a quick method development for the CCC separation of small molecules. CCC will then be able to be used in research facilities of any pharmaceutical industry as a complementary tool rather than as an emergency separation method for particularly difficult applications.

2. Experimental

2.1. Apparatus

An analytical high-performance counter-current chromatography (HPCCC) instrument, Mini-DE from Dynamic Extractions (Slough, UK), was used in this study. It was equipped with a coil of 17.7 ml with 0.8 mm bore tubing. The rotational speed was 2100 rpm (240 g). The Mini-DE was connected to either a Waters 2697 high performance liquid chromatography (HPLC) or an Agilent HP1100 HPLC. Both HPLC systems had quaternary pumps for mixing solvents on demand.

Samples were analyzed by a Waters 2695 HPLC equipped with 2996 photodiode array detector and Empower Pro workstation (Waters, USA). A Symmetry C_{18} column (75 mm × 4.6 mm I.D., 3.5 μm) (Waters, USA) was used for all analysis.