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

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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

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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.

2.2. Materials and reagents

Two model mixtures AZ Mix 1 and 2 used in this study are given in Tables 1 and 2 respectively. All materials were purchased from Sigma–Aldrich. All analytical grade solvents such as heptane, methanol and ethyl acetate for HPCCC separations and HPLC grade acetonitrile for analysis were supplied by Fisher Chemicals (Loughborough, UK). Deionised water and HPLC water were purified from a Purite Select Fusion pure water system (Thame, UK).

2.3. Measurement of partition coefficients

In the present study, the partition coefficients (K_D) for the target compounds were measured by HPLC as follows. Two-phase solvent systems with different ratios of heptane, ethyl acetate, methanol, and water were prepared in a test tube. Approximately 1–2 mg of each compound was dissolved in equal volumes of aqueous and organic phases of the thoroughly equilibrated two-phase solvent system. After the distribution equilibrium was established, an equal volume of upper and lower phases (200 μl) each was transferred into a separate vial and diluted with methanol (800 μl). Afterwards, both upper (organic phase) and lower (aqueous phase) were analyzed separately by HPLC. The partition coefficient was defined as $K_D = A_{upper}/A_{lower}$, where A_{upper} and A_{lower} were HPLC peak areas of each compound in the upper and lower phases respectively.

2.4. CCC separation

Three different approaches were used to make solvent systems containing the chosen ratios of heptane, ethyl acetate, methanol and water. The first approach involved making the system in the classical way by mixing solvents in a separatory funnel followed by equilibrating over night and then separating shortly before CCC separation. The second approach was to make each phase separately using ratios from [48]. The third approach involved mixing solvents on demand using quaternary pump with ratios from [48].

For each CCC separation, the coil was filled with the stationary (upper) phase in the head to tail mode. Then the mobile (lower) phase was pumped into the coil at a flow rate of 1 ml/min with a centrifuge rotational speed of 2100 rpm at 30 °C. When hydrodynamic equilibrium was established, the sample solution was injected into the coil through a 50 μl sample loop. Fractions were collected every minute from the start of each injection. To demonstrate the reproducibility most of the runs were repeated twice. The results were in good agreement. Although, it is important to control temperature of solvents used while mixing phase system on demand at the pump.

2.5. HPLC analysis and identification of CCC fractions

The model samples and CCC fractions were analyzed by HPLC using the same column and the same mobile phase. Separation was carried out at 40 °C with a binary mobile phase consisting of 0.1% aqueous trifluoroacetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1.0 ml/min. The gradient elution program for AZ Mix 1 was as follows: 0–6 min, 35–95% B; 6–8 min, 95% B. The gradient elution program for AZ Mix 2 was as follows: 0–7 min, 5–80% B; 7–8 min, 80% B.

3. Results and discussion

3.1. Measuring log K_D of AZ Mix 1 and 2 in different HEMWat solvent systems

HEMWat mixtures are the most studied of the quaternary two-phase systems. There are solvent ratio data for upper and lower phases measured by gas chromatography [48,49]. It gives the option to make phases separately or mix them at the pump on demand. The latter was proposed by Harris some time ago [50]. This is one of the important innovations in modern analytical and semi-preparative CCC. It reduces solvent consumption and eliminates the problem of degrading ethyl acetate in the presence of water forming acetic acid and ethanol as bi-products over a period of time [49].

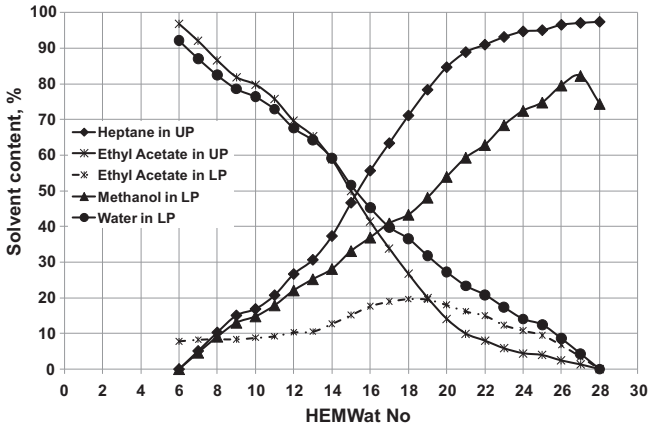

Fig. 1. Solvent ratio in the lower phases of HEMWat systems plotted on the basis of data from [48].

The organic phase in HEMWat 6–26 mainly consists of heptane and ethyl acetate while the aqueous phase consists of methanol and water with an almost constant amount of ethyl acetate. The polarity difference between phases remains the same while the overall polarity of the phase system decreases from 6 to 26. This arrangement gives an option to run both normal and reversed phase gradients by performing ethyl acetate and methanol gradients respectively. However, from the solvent ratios in the lower aqueous phase plotted on the basis of data from [48] (Fig. 1), the most interesting range appears to be between 8 and 26, or even 22 where methanol and water ratios change evenly. Therefore, this range of systems has been chosen for further studies.

The first step in this study was to measure K_D values as accurately as possible for all compounds from the two model mixtures. The first model mix, AZ Mix 1 represents a set of 7 compounds used at AstraZeneca to aid HPLC conditions selection for compound purification. Since the aqueous phase of the freshly made HEMWat systems has pH 5.5–6, most of AZ Mix 1 compounds are neutral spanning a range of octanol *C*log *P* of 1.5–4 (Table 1). The exceptions are warfarin (3), which will be 80% anionic (pK_a acid 4.9), and dipyridamole (1), which will be 89% cationic (pK_a base 6.4). Log K_D values were determined for each compound in HEMWat systems 8, 14,17,20, 22 and 26 using the shake flask method according to the procedure described in [1]. Plotting log K_D against HEMWat numbers, where the latter is a surrogate measure of overall system solvent polarity, reveals linear correlations for the HEMWat range between 16 and 22 and overall non-linear correlations with a third degree polynomial function as the best fit for the whole HEMWat range from 8 to 26 (Fig. 2a). The slope of these plots is seen to correlate with the polarity of the compound with molecules having the lowest octanol log *P* and most H bond donors and acceptors showing the steepest dependence (dependence is greater on donor count than acceptor). This is believed to reflect the changing nature of the HEMWat system with the solvent switching from predominantly ethyl acetate in the upper phase to predominantly hydrocarbon in the upper phase on going from HEMWat 8 to 26.

Abraham has developed linear free energy relationships (LFERs) characterizing the partitioning of compounds in both the ethyl

Table 1
Physical properties of compounds from AZ Mix 1.

No	Common name	*C* log *P*	ACD log *P*	ACD log *D* (7.4)	AZ log *D* (7.4)	*n* donors	*n* acceptors	A Leo log *P** oct	Lit log *P*16	Molecule ion class
1	Dipyridamole	1.49	−1.22	−2.62	0.96	4	12			Neutral
2	Methyl-4-amino-3-methyl benzoate	1.84	1.87	1.87	1.64	2	3			Neutral
3	Warfarin	2.9	3.42	0.52	1.18	1	4	2.7		Acid (pK_a 4.94)
4	Methyl 2-acetamido-5-bromobenzoate	2.55	2.77	2.77	2.46	1	4	1.08		Neutral
5	Naphthalene	3.32	3.45	3.45	2.92	0	0	3.3	3.41	Neutral
6	Phenanthrene	4.49	4.68	4.68	3.43	0	0	4.46	4.74	Neutral
7	Biphenyl	4.03	3.98	3.98	3.75	0	0	4.01	4.08	Neutral

Table 2
Physical properties of compounds from AZ Mix 2.

No	Drug name	*C* log *P*	ACD log *P*	ACD log *D* (7.4)	AZ log *D* (7.4)	*n* donors	*n* acceptors	A Leo log *P**	AZ Meas log *D* (7.4)	Ion class
8	Atenolol	−0.11	0.1	−1.67	−1.3	4	5	0.16	−1.65	Base
9	Thiamphenicol	−0.1	−0.27	−0.27	−0.41	3	6	−0.27	−0.41	Neutral
10	Pentoxifylline	0.12	0.32	0.32	0.53	0	7	0.29	0.02	Neutral
11	Cinoxacin	1.74	0.36	−5.19	−1.21	1	7		0.5	Acid
12	Griseofulvin	2.05	3.53	3.53	2.13	0	6	2.18	2.3	Neutral
13	Tolbutamide	2.5	2.34	2.34	−0.22	2	5	2.34	0.395	Acid
14	Albendazole	3.46	3.07	3.06	3.11	2	5		3.51	Neutral
15	Glyburide	4.24	3.75	3.75	1.5	3	8	3.08	2.16	Acid
16	Diethylstilbestrol	4.96	5.93	5.93	3.41	2	2	5.07	3.9	Neutral

acetate/water system and hydrocarbon/water systems (see Eqs. (1) and (2)) [51]. Looking at the coefficients of these equations the greatest difference between them is for the hydrogen bond acidity term (α) reflecting that as the relative proportions of ethyl acetate to hydrocarbon change in the HEMWat system hydrogen bond donating ability of a compound will have a significant impact on the log K_D.

$$\log P_{\text{Ethyl acetate}} = 0.25 + 1.16R_2 - 1.40\pi_2^H - 0.05\alpha_2^H - 3.76\beta_2^H + 3.74V \quad (1)$$

$$\log P_{\text{Alkane}} = 0.29 + 0.65R_2 - 1.66\pi_2^H - 3.52\alpha_2^H - 4.82\beta_2^H + 4.28V \quad (2)$$

However log K_D values around 0 can be achieved across the HEMWat range 8–26 (Fig. 2a) suggesting that a gradient system spanning this range could potentially enable compound separation of samples like the ones in the AZ Mix 1 set.

Compounds in the second model mix, AZ Mix 2, were selected to embrace a diverse range of drug molecules spread across a *C* log *P* range of −0.1 to 5.0 including acids, bases and neutrals (Table 2). In chosen HEMWat systems, most of the compounds are neutral except base atenolol (8), which will be fully cationic, and acids cinoxacin (11) (91% anionic, pK_a 4.5), glyburide (15) (67% anionic, pK_a 5.2) and tolbutamide (13) (61% anionic, pK_a 5.3). Similar correlations of the linear range for HEMWat 16–22 and the third order polynomial for overall range were observed between log K_D and the HEMWat number (Fig. 2b). Again, log K_D values around 0 can be achieved for all the compounds except atenolol (8) and cinoxacin (11) suggesting that a gradient system spanning this range could potentially separate AZ Mix 2 but the most polar compounds, atenolol (8) and cinoxacin (11), would elute at the solvent front.

Moreover, there is the suggestion of a link to octanol log *P* when analyzing the log K_D values of the compounds individually. However these correlations are too weak, indicating that octanol–water is too different a solvent system to that of HEMWat to be an effective model.

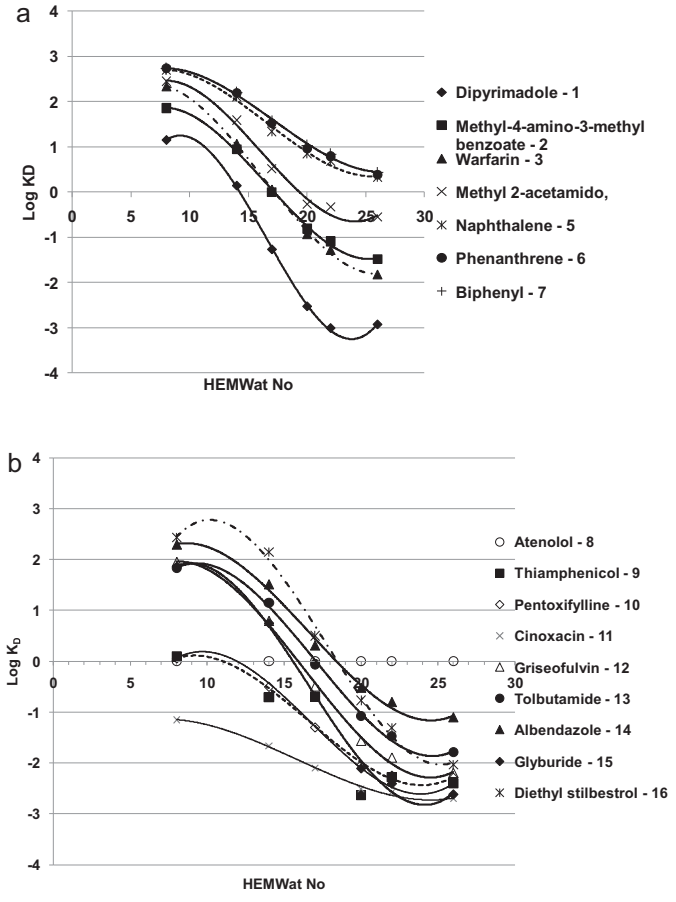

Fig. 2. Correlation between log K_D for AZ Mix 1 (a) and AZ Mix 2 (b) and HEMWat system numbers.

3.2. Gradient separation of AZ Mix 1 and 2

Based on log K_D values the first linear gradient for AZ Mix 1 was run with HEMWat 17–26 in reversed phase mode in 30 min (data are not shown). For all experiments the HPCCC column was first equilibrated by starting the system in reversed phase mode. Then 50 μl of sample was injected onto the column. The isocratic elution was held for one displaced volume, which is equal to the volume of

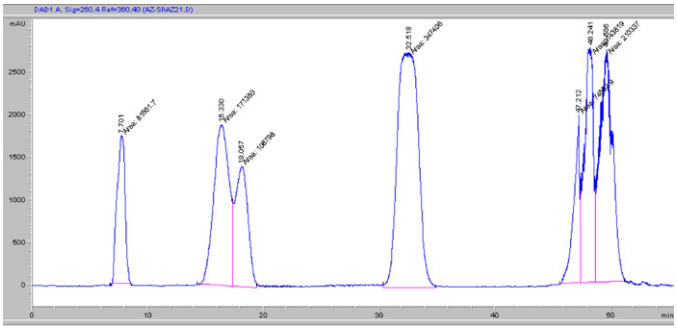

Fig. 3. Gradient elution chromatogram for AZ Mix 1. Mini HPCCC, 17.7 ml, 0.8 mm bore, 2100 rpm, 1 ml/min. Gradient start at 7 min from HEMWat LP 17 to LP 28 in 30 min.

stationary phase displaced from the column during hydrodynamic equilibrium plus the volume of the inlet and outlet leads, followed by 30 min linear gradient of lower phase. To mimic the screening conditions that would be used in industry, both stationary and mobile phases were mixed on demand. The AZ Mix 1 sample solution was made up in DMSO. All compounds eluted within 62 min. As expected, dipyrimadole (1) eluted at the solvent front followed by partly resolved methyl-4-amino-3-methyl benzoate (2) and warfarin (3), and then methyl 2-acetamido 5-bromo-benzoate (4). However, the three most non-polar compounds, naphthalene (5), phenanthrene (6) and biphenyl (7), co-eluted with very little separation. This might be caused by too small a change in polarity and a quick loss of stationary phase. Therefore, the gradient was extended to HEMWat 17–28 (Fig. 3). In these conditions, stationary phase retention (S_f), equal to a ratio of stationary phase volume to a column volume, changed from the initial 73.4% to the final 19.8%. As a result, all the compounds eluted in just under 52 minutes with the same resolution as in the previous run, but with the non-polar components this time partly separated.

The AZ Mix 2 is more diverse in terms of structure and properties than AZ Mix 1 and therefore, solubility issues could be a problem. To investigate how elution times are affected by using different solvents for the sample solubilisation, the AZ Mix 2 was first dissolved in DMSO (Fig. 4a) and then in MeOH (Fig. 4b). Both separations were carried out in isocratic mode with HEMWat 17 in reversed phase with mobile mixed on demand by the quaternary pump. The introduction of 50 μl of both DMSO and MeOH had similar effect on stationary phase retention, which decreased from the initial 73.5% to the final 56.5%. It can be seen that there is very little difference between the two chromatograms in Fig. 4. The only compounds affected are those eluting before $K_D = 1$ (before 20 min). All the others elute at the same time position. Furthermore, the presence of DMSO creates its own short gradient and helps the separation of two very polar compounds eluting with the solvent front. Therefore, 50 μl of the sample solution made up in a solvent foreign to the HEMWat system injected into a 17.7 ml analytical HPCCC column did not affect the separation too much.

Three reversed phase linear gradients were applied to the AZ Mix 2. The first HEMWat gradient from No 8 to 17 is shown in Fig. 5a (the initial S_f was 52%). There are clearly three groups of compounds in AZ Mix 2. The first polar group containing atenolol (8) and cinoxacin (11) elutes with the solvent front according to their K_D values. There is no HEMWat without pH modifiers added capable of separating these compounds. The second group, thiamphenicol (9) and pentoxifylline (10), elutes about $K_D = 1$, which corresponds to the static K_D values (Fig. 2b). It is questionable, which system can be used to separate this pair as their K_D values are so similar for all HEMWat systems tested. Compounds albendazole (14), diethylstilbestrol (16), glyburide (15), griseofulvin (12) and tolbutamide

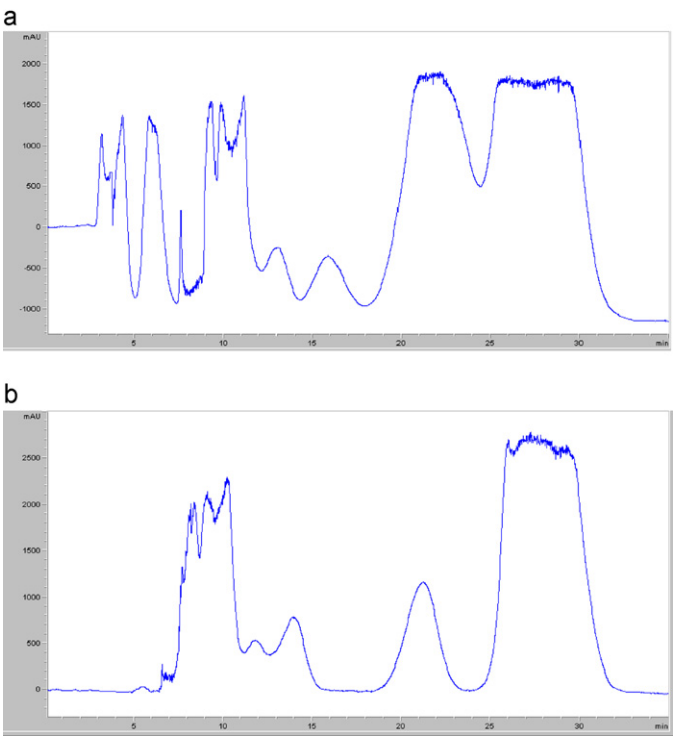

Fig. 4. Isocratic elution chromatogram for AZ Mix 2 dissolved in DMSO (a) and MeOH (b). Mini HPCCC, 17.7 ml, 0.8 mm bore, 2100 rpm, 1 ml/min, HEMWat 17 RP.

(13) can be partly separated by this solvent system and this can be clearly seen from Fig. 5.

Moving to the next range of gradients from HEMWat 17 to 22 (Fig. 5b) resulted in complete separation of compounds albendazole (14) and diethylstilbestrol (16) with the initial S_f of 73.4%. However, a strange effect was observed for compounds glyburide (15), griseofulvin (12) and tolbutamide (13) eluting in two portions. Perhaps it can be explained by their ionisable nature as glyburide is 67% anionic (pK_a 5.2) and tolbutamide is 61% anionic (pK_a 5.3) due to lack of pH control and the vastly changing K_D values between HEMWat 14 and 17. This phenomenon disappears when the gradient was widened from 14 to 22 providing 51% of the initial S_f (Fig. 5c). In this case, the first two groups completely co-elute but in the third non-polar group the majority are separated. However, the solvent ratio of the mobile phase when compounds eluted from the column did not correspond to the solvent system providing their K_D of around unity.

Further extending the gradient from HEMWat 8 to 22 in 30 min (data not shown) and in 60 min led to elution of all the compounds within 70 min with the initial S_f of 52% but no stationary phase left afterwards (Fig. 6). Again, the compounds eluted partly separated in three groups.

3.3. Correlation between gradient elution and HEMWat system

Although both AZ Mix 1 and Mix 2 can be partly or completely separated by gradient elution with HEMWat systems by varying a gradient range, there is still the need to establish a link between the gradient run and the solvent system suitable for separation of a target compound. The approach described below might offer a potential solution or, at least, the first step towards it.

First of all, the correlation between stationary phase retention and the elution time/volume (V_R/t_R) of the compounds should be established. Recording an initial volume of the stationary phase (V_s) before starting the gradient (this can be calculated since the system total volume is known and the displaced volume of stationary phase

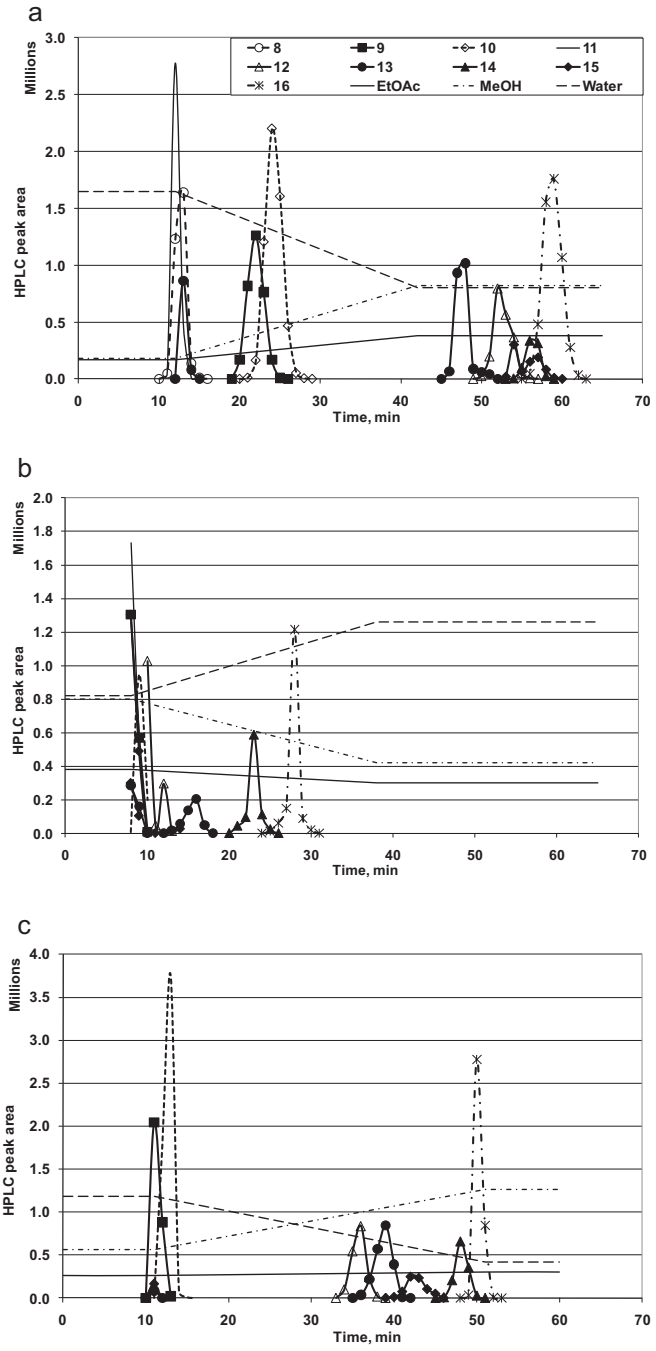

Fig. 5. Gradient elution fractograms for AZ Mix 2 constructed from HPLC analysis of HPCCC fractions. Mini HPCCC, 17.7 ml, 0.8 mm bore, 2100 rpm, 1 ml/min. Gradient HEMWat LP 8–17 (a), LP 17–22 (b) and LP 14–22 (c) in 30 min.

($V_d = V_m + V_{in} + V_{out}$) measured during equilibrating) and the final V_s after emptying a column will allow one to plot V_s against run time or elution time/volume t_R/V_R. We assume that this is linearly dependency on time as the mobile phase gradient is linear. Ignore for now any change in stationary phase composition with time. A trend line equation can be calculated:

$$V_s = m_1 \times V_R + c_1 \quad (3)$$

where m_1 is the mathematical gradient and c_1 is the intercept.

This simple equation can be used to correlate any elution time during the gradient run to the stationary phase volume at that particular moment and is valid for $V_R > V_d$.

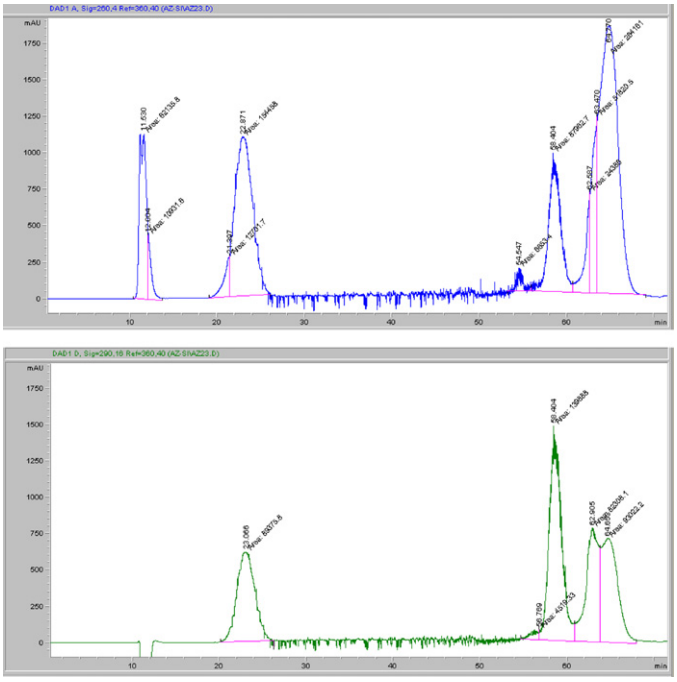

Fig. 6. Gradient elution chromatogram for AZ Mix 2. Mini HPCCC, 17.7 ml, 0.8 mm bore, 2100 rpm, 1 ml/min. HEMWat LP 8–20 in 60 min.

Afterwards, using Eq. (4) from CCC theory [52] K_D can be calculated:

$$K_D = \left(\frac{V_R - V_c}{V_s}\right) + 1 \quad (4)$$

where V_c is the column volume, V_R the retention volume of the compound and V_s is the volume of stationary phase retained in the column.

The final step is to link K_D of the compound from the gradient run to the HEMWat number using plots $\log K_D$ against HEMWat number based on K_D values measured by the shake flask method in static conditions as described earlier (Fig. 2). Again, each line (compound) can be characterised by a trend line with the appropriate equation. The latter should be used to calculate the HEMWat number.

For demonstrating how this approach works, it has been applied to AZ Mix 1 and Mix 2 with the assumption that $\log K_D$ against HEMWat is a linear correlation between HEMWat 14 and 20.

$$\log K_D = m_2 \times \text{HEMWat No} + c_2 \quad (5)$$

where m_2 is the mathematical gradient and c_2 is the intercept for the linear fit.

The results of the estimation are given in Tables 3 and 4. Row 7 represents the HEMWat No determined by the approach described above using Eqs. (3)–(5). Row 8 contains HEMWat No calculated from Eq. (5) assuming that $K_D = 1$. Using the linear correlation of $\log K_D$ vs. HEMWat number this approach worked very well for HEMWat 14–20 with the good comparison of HEMWat number determined from gradient retention time and that using $K_D = 1$ for compounds eluting just under 2 column volumes. However, the more non-polar compounds eluting later gave a poorer comparison of HEMWat number using the two methods. This can be explained by loss of stationary phase in the column and by deviation from linearity of the $\log K_D$ vs. HEMWat number plot in this lipophilica region. The third order polynomial equation gives a better fit of $\log K_D$ vs. HEMWat number but requires more sophisticated mathematical approach with iterative programming.

Table 3
Estimation of HEMWat system number based on gradient log K_D for AZ Mix 1.

No	Log $K_D = m_2 \times$ HEMWat No + c_2 from graph	Dipyrimadole (1)	Methyl-4-amino-3-methyl benzoate (2)	Warfarin (3)	Methyl 2-acetamido, 5-bromo-benzoate (4)	Naphthalene (5)	Phenanthrene (6)	Biphenyl (7)
1	Elution volume, V_R (ml)	7.7	16.33	18.06	32.52	47.21	48.24	49.59
2	V_s (ml)	12.92	11.31	10.99	8.29	5.55	5.36	5.11
3	K_D	0.23	0.88	1.03	2.79	6.32	6.70	7.25
4	Log K_D	−0.65	−0.06	0.01	0.45	0.80	0.83	0.86
5	c_2	3.2747	3.4848	4.29	3.86	3.8555	3.93	3.8923
6	m_2	0.2626	0.2007	0.25	0.185	0.1413	0.14	0.1361
7	HEMWat No using Eqs. (3)–(5)	14.9	17.6	17.1	18.5	21.6	22.2	22.3
8	HEMWat No calculated from Eq. (5) if $K_D = 1$	12	17	17	21	27	28	29

Table 4
Estimation of HEMWat system number based on gradient log K_D for AZ Mix 2.[a]

No	Log $K_D = A - B \times$ HEMWat No from graph	Thiamphenicol (9)	Pentoxifylline (10)	Tolbutamide (13)	Griseofulvin (12)	Albendazole (14)	Glyburide (15)	Diethylstilbestrol (16)
1	Elution volume, V_R (min)	21.4	22.87	54.55	58.4	62.59	63.47	64.65
2	V_s (ml)	7.96	7.77	3.60	3.09	2.54	2.42	2.27
3	K_D	1.46	1.67	11.24	14.17	18.68	19.89	21.71
4	Log K_D	0.17	0.22	1.05	1.15	1.27	1.30	1.34
5	c_2	3.0272	2.5782	5.701	5.4646	5.4461	6.3613	8.0993
6	m_2	0.2523	0.2247	0.3324	0.343	0.2916	0.4097	0.436
7	HEMWat No using Eqs. (3)–(5)	11.3	10.5	14.0	12.6	14.3	12.4	15.5
8	HEMWat No calculated from Eq. (5) if $K_D = 1$	12	11	17.2	15.9	18.7	15.5	18.6

[a] Compounds are listed in the elution order.

4. Conclusions

This study is the first attempt at establishing a correlation between K_D values, HEMWat system number and elution profile when using a gradient in CCC. It has been demonstrated that the prediction of the solvent system on the basis of a general gradient is possible by using three simple steps. However, it requires considerably more refinement before it becomes a more widely available tool.

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