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Short communication

The effect of eluent pH and compound acid–base character on the design of generic-gradient reversed-phase high-performance liquid chromatography (RP-HPLC) methods for use in drug discovery

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Abstract

The design of generic reversed-phase high-performance liquid chromatography (RP-HPLC) gradient methods for the analysis of compound mixtures or 'cocktails' has been investigated with particular reference to the eluent pH and the type of compound (acid, base or neutral) analysed. The use of eluents with an acidic eluent pH, an approach which is widely employed, can lead to non-retention of polar bases resulting in 'failure' of the method. This problem is aggravated where the majority of compounds submitted for analysis are bases, which is typical of many drug discovery programs. The problem can be ameliorated through the use of eluents with near neutral pH. Although these neutral pH eluents can lead to co-elution when cocktails are analysed and possibly ion-suppression where mass spectrometry (MS) is the detection method, this can be avoided through optimisation of the gradient shape.

Keywords: Acid–base character; RP-HPLC

1. Introduction

The measurement of physicochemical, metabolic or biopharmaceutical properties of drug candidates typically involves the use of gradient reversed-phase high-performance liquid chromatography (RP-HPLC) [1–4]. In an endeavour to eliminate method development time and maximise throughput, many pharmaceutical laboratories are employing generic HPLC methods. In order to create further gains in efficiency through reduction in total analysis time, compounds are often tested or analysed as mixtures or 'cocktails'. Typically several compounds would be dosed simultaneously to an animal or, following an in vitro incubation of single compounds, a number of compounds may be pooled or 'cocktailed' prior to analysis.

Any successful generic method should be able to chromatograph and elute a wide range of compound types with good performance, whilst giving the best separation between any two compounds selected at random. Although most workers are using mass

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spectrometry detection with the implication of good selectivity, good quality separations are still required if problems associated with co-elution and ion suppression [5–8] are to be avoided.

Whilst most laboratories have preferred column/eluent combinations, there has been limited study on the design of generic HPLC methods [9–11] for use in drug discovery. Although the effect of eluent pH in HPLC separations can be considered obvious, we have attempted through simulations, to quantify the risk of the analysis failing, based on the eluent pH and the acid/base nature of the compounds submitted for analysis.

2. Experimental

The C log P (octanol/water) data used in this study were calculated [12] for around 600,000 compounds in the collection at Alderley Park. The data was normally distributed with a mean C log P of 3.27 and a standard deviation of 2.31. Marketed oral drug compounds tend to have a low log P [13] and hence it is not unexpected that this mean is higher than the value of 1.73 observed for a small set of marketed drugs [14] and other collections of 'drug-like' molecules [15]. The distribution of acid/base dissociation constants (pK_a) were taken from the results of compounds submitted for pK_a determination in our laboratory. The distribution for both sets of compounds, which were highly skewed, are shown in Fig. 1a and b. The assumption was made that these were representative of all acids/bases in the compound collection.

All data analysis and simulations were performed using Microsoft Excel.

HPLC was carried out using an Agilent 1100 system, the column was 100 mm × 2 mm i.d. packed with Phenomenex Prodigy ODS3 (Phenomenex, Macclesfield, UK). The eluent consisted of ammonium acetate (50 mM) in water (pH 6.8) and methanol with a gradient being run from 5 to 95% methanol over 15 min with a 6 min hold at 95% methanol, using a flow rate of 0.3 ml/min. The gradient shape was linear although the separation had been enhanced by the use of a large injection volume of a sample (1 ml) containing a low concentration of acetonitrile (6.6%). This strategy was adopted since it allows high mass loading [16] without affecting peak shape, but it also gives what is in effect a prolonged isocratic start to the separation.

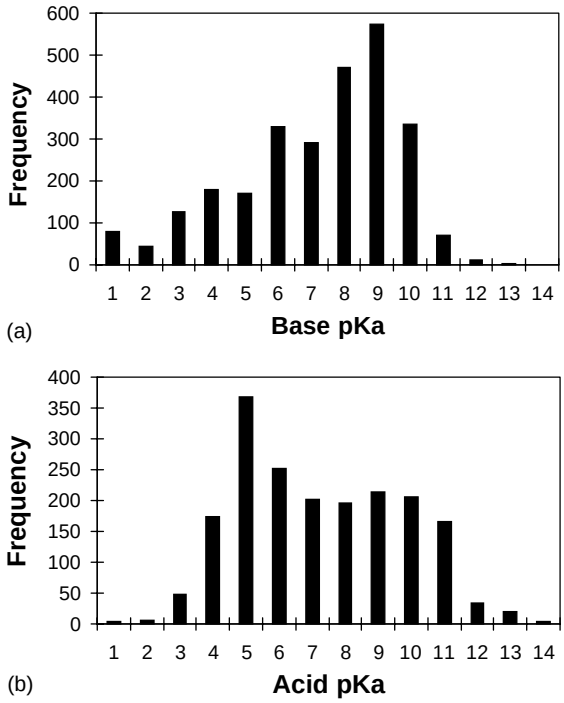

Fig. 1. The measured pK_a for bases (a) and acids (b) taken from the company database.

3. Results and discussion

Fig. 2 shows the relationship between retention time and log D for 1258 compounds (acids, bases and neutrals) chromatographed using the HPLC system described above. The log D was calculated from the C log P [12] and the pK_a was estimated using standard relationships [17]. Whilst there is a high degree of scatter in the data there is an underlying linear relationship between retention time and log D. This is expected since the major retention mechanism in reversed-phase HPLC is partitioning. It is possible that the quality of this relationship could be improved by taking into account the variation in eluent pH across the gradient brought about by the effect of the increasing methanol concentration and the modification of the analyte pK_a due again to the effect of the methanol. Whilst both these phenomena can be

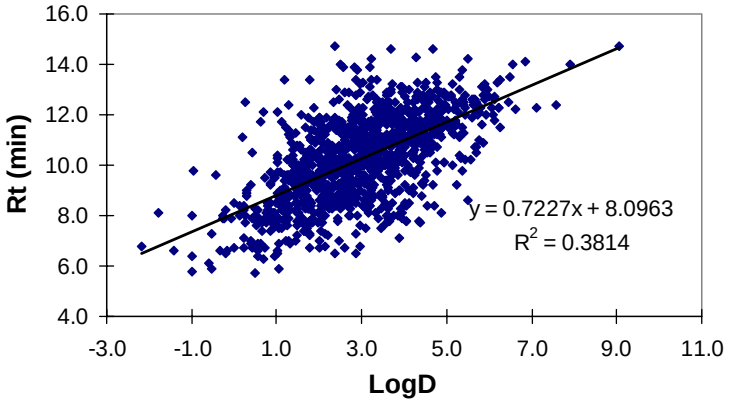

Fig. 2. The relationship between retention time and log *D* for 1258 mixed compounds.

modelled [18,19], the precision of the prediction above 80% methanol is poor [18] and the more complex analysis is unlikely to affect the outcome.

It would appear therefore, that log D, which is easily calculated, can be used as a surrogate for retention time in a range of simulations, thus avoiding the need to chromatograph a very large number of compounds under a variety of conditions. In practice, compounds with a log D < -2 are either unretained or are eluted with highly distorted peak shapes on the described HPLC system. A log D of –2, equivalent to a retention time of around 5 min is therefore considered to be a practical lower limit of retention for the described method.

It is also interesting to note that this particular HPLC method is capable of chromatographing compounds covering a wide range of lipophilicity of at least eight log D units.

Using the distributions of C log P and measured pK_a described above, it is possible to simulate log D data for a large group (10,000) compounds having differing proportions of acids, bases and neutrals. Using this simulated data set and the proposed relationship between retention time and log D (Fig. 2), it is thus possible to study the effect of eluent pH on compound retention.

Table 1 shows the proportion of compounds with log D < -2, i.e. those which would not be retained,

Table 1
The percentage of compounds which fall below the cut off equivalent to a log $D = -2$ (retention time of 5 min) for seven different sets of compounds ($n = 10{,}000$) made up of different proportions of acids, bases and neutrals, chromatographed at different pHs

Eluent pH	Ratio of acids:bases:neturals in the data set						
	33:33:33	10:20:70	15:30:55	20:20:60	100:0:0	0:100:0	0:0:100
10.0	9.8	3.7	5.0	6.2	27.0	1.2	1.1
9.0	7.0	2.9	3.8	4.5	18.2	1.5	1.1
8.0	4.9	2.4	2.9	3.4	11.4	2.1	1.1
7.0	3.8	2.4	2.8	2.9	6.2	4.0	1.1
6.5	3.7	2.4	2.9	2.7	4.6	5.5	1.1
6.0	3.9	2.6	3.4	2.8	3.3	7.7	1.1
5.0	5.6	3.6	5.0	3.7	1.9	13.8	1.1
4.0	8.4	5.5	7.6	5.5	1.4	22.7	1.1
3.0	11.8	7.7	10.8	7.7	1.2	33.5	1.1
2.5	13.7	8.9	12.5	8.9	1.2	39.2	1.1
2.0	15.6	10.0	14.1	10.0	1.2	44.9	1.1

versus different eluent pH values, for the simulated data sets. The pH values used in these simulations span the range normally employed in routine HPLC. This includes pH 2.4 as obtained with formic acid (0.2% or 53 mM) through pH of around 7 for 50 mM ammonium acetate or formate to a pH of 10 as would be achieved with dilute ammonia. The use of high pHs is only recommended with stationary phases which have been specifically designed to be stable under such conditions.

It can be clearly seen that the major problem relates to the chromatography of basic compounds. This is particularly so for compound collections comprising a high proportion of bases, when analysed with acidic eluents (pH < 6). Similarly, though less marked problems also occur with compound sets containing a high proportion of acids when chromatographed at high pH (>6.5). To avoid these problems an eluent pH of around 6 to 7 would appear to be ideal since this reduces the proportion of non-retained compounds to <3% even with a relatively high proportion of bases (∼30%).

An eluent pH around neutral is a useful overall compromise since it minimises non-retention of both acids and bases. Furthermore, with mass spectrometry as the method of detection, then ionisation (and hence sensitivity) will be favoured when elution occurs with more highly organic eluents. Therefore, eluent conditions, such as high or low pH which result in elution of compounds in water rich eluents are also to be avoided from a sensitivity point of view. The counter argument to the use of neutral pH eluents is that they will tend to produce less discriminatory separations with a significant degree of co-elution.

There are currently no reliable methods for calculating large numbers of pK_as, thus it is difficult to estimate the exact proportion of acids, bases and neutrals in a particular company's collection. However, less rigorous analysis carried out using the SMARTS language [20] to identify compounds ionised at physiological pH, suggests that in the current Alderley Park compound collection, there are around 20% bases and 11% acids. An eluent pH of 6.8 as obtained using ammonium acetate would appear to be optimal for this collection of compounds.

The optimum eluent pH for a generic RP-HPLC method is a balance between failure to retain (analyse) compounds and a failure to separate compounds. The former, however, would appear to be the more important since the latter problem can be ameliorated by manipulating the shape of the chromatographic gradient. This is an approach we have used successfully in our laboratories. In 1998, we carried out in vivo studies on 1851 compounds using the generic RP-HPLC–MS methodology. Of these, 1768 (95.5%) were detected and a retention time recorded. Of the remaining 4.5%, around three quarters of these were either unstable or the wrong compound had been submitted for analysis. For only four compounds (0.2% of the total) could the non-detection be unequivocally attributable to chromatography problems, either late or early elution, or poor peak shape.

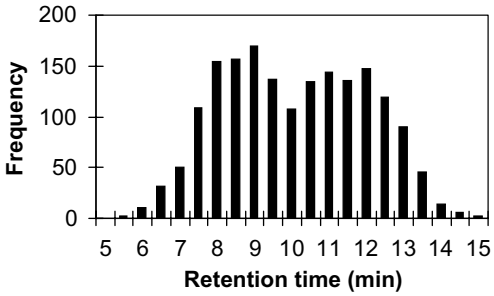

Fig. 3. Retention distribution for 1768 compounds chromatographed with an eluent having a pH of around 7 and with an optimised gradient to maximise compound separation.

The distribution of retention times for the 1786 compounds is shown if Fig. 3. Through the use of an eluent with a pH of 6.8, we have avoided the problem of early elution and as a consequence non-retention. Furthermore, by adjustments to the gradient shape, effectively introducing a more shallow rise the middle, the distribution of retention has been evened out thus avoiding excessive co-elution and potential ion suppression.

4. Conclusions

The successful application of generic gradient RP-HPLC methods in drug discovery involves a clear understanding of the goals: maximal separation thus avoiding ion suppression effects or maximal success in retaining and analysing compounds. Having decided on the latter, then it is necessary to have an understanding of the distribution of acids, bases and

neutrals in the target compound collection and to adjust the eluent pH to match this set of compounds. Where there is a large percentage of bases then the use of an eluent with near neutral pH is recommended. For a compound collection made up of approximately 20% bases such as that at Alderley Park, then the use of a methanol eluent containing ammonium acetate with a pH of 6.8 has proved highly successful. It is important however to continuously monitor the success of such methods as the shape of the compound collection or current chemistry changes.

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References

[1] J. Ayrton, R. Plumb, W.J. Leavens, D. Mallett, M. Dickins, G.J. Dear, Rapid Commun. Mass Spectrom. 12 (1998) 217.
[2] R. Plumb, G.J. Dear, D. Mallett, I.J. Fraser, J. Ayrton, C. Ioannou, Rapid Commun. Mass Spectrom. 13 (1999) 865.
[3] L. Romanyshyn, P.R. Tiller, C.E.C.A. Hop, Rapid Commun. Mass Spectrom. 14 (2000) 1662.
[4] F. Leroy, B. Presle, F. Verillon, E. Verette, J. Chromatogr. Sci. 39 (2001) 487.
[5] B. Law, D. Temesi, J. Chromatogr. B. 748 (2001) 21–30.
[6] D.L. Buhrman, P.I. Price, P.J. Rudewicz, J. Am. Soc. Mass Spectrom. 7 (1996) 1099–1105.
[7] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.
[8] F. Beaudry, J.C.Y. Le Blanc, M. Coutu, N.K. Brown, Rapid Commun. Mass Spectrom. 12 (1998) 1216–1222.
[9] I.M. Mutton, Chromatographia 47 (1998) 291.
[10] Y-F. Cheng, Z. Lu, U. Neue, Rapid Commun. Mass Spectrom. 15 (2001) 141.
[11] C. Seto, K. Bateman, B. Gunter, J. Am. Soc. Mass. Spectrom. 13 (2002) 2.
[12] Medchem Software Manual, version 3.4, Pomona College, Claremont, CA, 1986.
[13] M.C. Wenlock, R.P. Austin, P. Barton, A.M. Davis, P.D. Leeson, J. Med. Chem. 46 (2003) 1250.
[14] T. Sakaeda, N. Okamura, S. Nagata, T. Yagami, M. Horinouchi, K. Okumura, M. Yamashita, M. Hashida, Biol. Pharm. Bull. 24 (2001) 935–940.
[15] T.I. Oprea, J. Comput. Aided Mol. Des. 14 (2000) 251.
[16] M.J. Mills, J. Maltas, W.J. Lough, J. Chromatogr. A. 759 (1997) 1.
[17] D.D. Perrin, B. Dempsey, E.P. Serjeant, pK_a Prediction for Organic Acids and Bases, Chapman and Hall, London, 1990.
[18] I. Canals, K. Valko, E. Bosch, A.P. Hill, M. Roses, Anal. Chem. 73 (2001) 4937.
[19] M. Roses, E. Bosch, J. Chromatogr. A. 982 (2002) 1.
[20] Available from Daylight Chemical Information Systems, http:/www.daylight.com.