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Optimisation of the enantiomeric separation of 12 2-aminotetralin analogues using Chiral AGP high-performance liquid chromatography by simultaneous factorial design

S.A. Wood [a,*], J.M. Long [a], R.J. Simmonds [a], J.W. Bridges [b], D. Stevenson [b]

[a] *Pharmacia and Upjohn, Fleming Way, Crawley, West Sussex RH10 2LZ, UK*
[b] *Robens Institute of Health and Safety, Surrey University, Guildford, Surrey GU2 5XH, UK*

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Abstract

A method for the simultaneous optimisation of mobile phase composition for the resolution of pairs of enantiomers of 12 2-aminotetralin analogues is presented. The selectivity necessary to discriminate between 12 analytes was obtained by using mass selective detection. The ability to examine more than a few analytes at a time extends the otherwise limited applicability of a factorial design strategy to the rapid development of chiral assays. © 1997 Elsevier Science B.V.

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1. Introduction

Pharmacologically active analogues of the 5-hydroxytryptamine receptor agonist 8-hydroxy-(di-*n*-propylamino)tetralin (8-OH-DPAT) [1] have been screened for metabolic stability by an in-vitro model using isolated rat hepatocytes [2,3] (see Fig. 1, compound **II**). Hepatocyte incubations were, for the most part, conducted on racemic mixtures, with subsequent quantitative analysis using achiral chromatographic techniques, [4,5] since these could be developed from a 'generic' high-performance liquid chromatography (HPLC) assay more quickly than enantioselective methods. If chiral methods of quantification could be developed more quickly, information regarding enantiomeric stability would become available to assist the development of new analogues, and improve the computational chemistry models being developed for this class of compound. The development of chiral quantification methods is, however, generally too slow to meet the deadlines common to screening programs. This arises because, at least in part, generic chiral assays are not available. Small changes in chemical structure often adversely effect resolution so that prediction of suitable conditions for a new analogue is not

* Corresponding author. Present address: British Biotech Pharmaceuticals, Watlington Road, Oxford OX4 5LY, UK.

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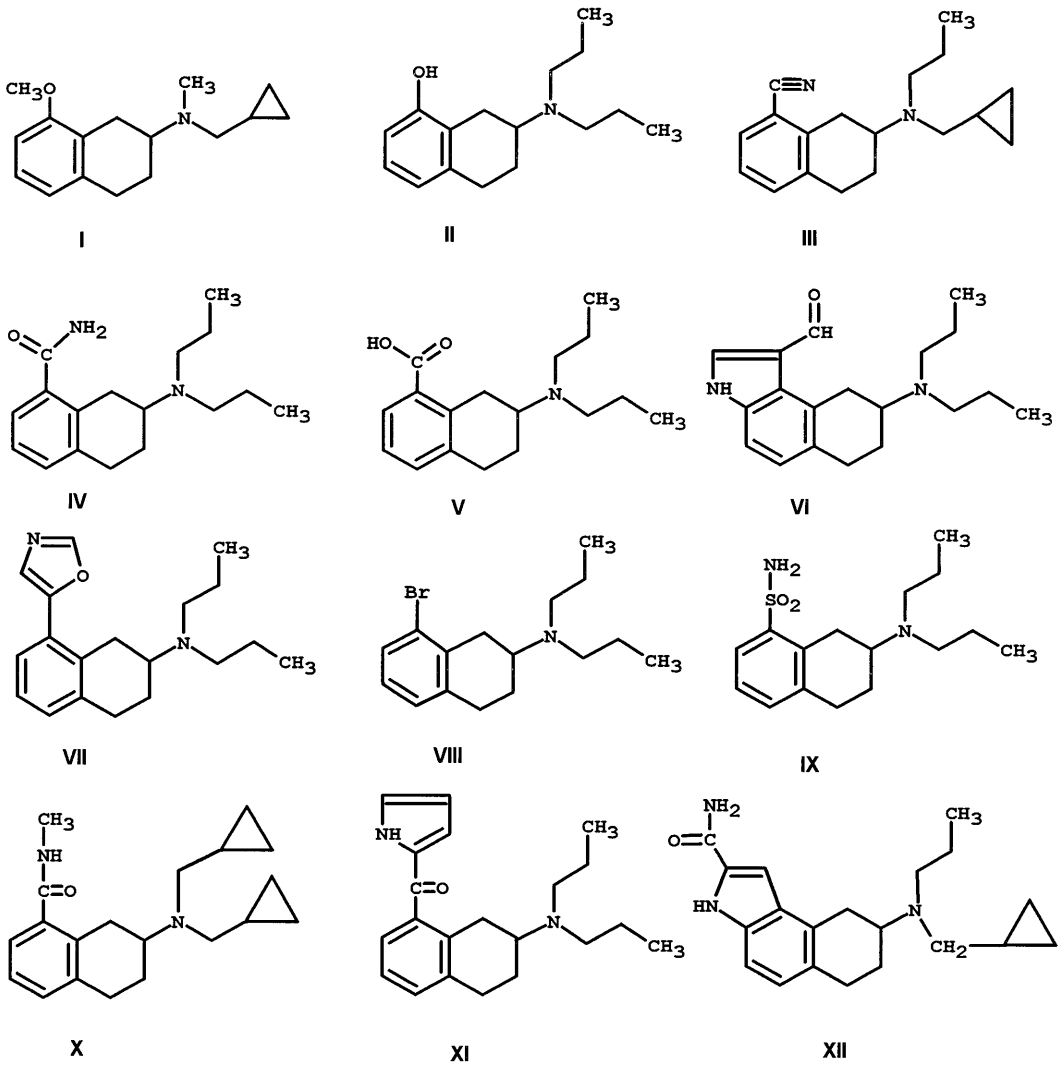

Fig. 1. Structures of the 2-aminotetralins.

possible, and each individual assay requires exploration of a number of variables that affect enantioselectivity.

Factorial [6], or composite factorial [7,8], experimental design strategies can assist in testing variables (called factors) for their effect upon experimental outcomes (in this instance enantioselectivity) and the dependence of factors upon each other. Factorial and composite factorial design experiments exploring the chiral separation of two of these compounds (**II** and **XI**) have been reported previously [9]. The composite factorial design was used so that it was not necessary to assume a linear response to changes in the factors, explicit when using the two level (e.g., 2^3) factorial design. This more complex strategy, however, did not produce any significant additional data. The experiments showed that whilst some insight into the mechanism of the chromatographic separation was obtained, a large number of chromatographic runs would be necessary if data on more than one analyte was required. In order to apply a factorial

Table 1
Mobile phase combinations for the simultaneous 2^3 factorial design

Expt. No.	Factor A: pH	Factor B: H bonding	Factor C: % organic
1	4	CH_3CN	3
2	7	CH_3CN	3
3	4	2-Propanol	3
4	7	2-Propanol	3
5	4	CH_3CN	8
6	7	CH_3CN	8
7	4	2-Propanol	8
8	7	2-Propanol	8

Table 2
Capacity factor and enantioselectivity for the 12 test compounds run individually and as a mixture (mobile phase: ammonium acetate (0.05 M, pH 7.0):2-propranol (92:8 v/v))

Compound no.	Individual runs		Mixture					
			k'			α		
	k'	α	Run 1	Run 2	Run 3	Run 1	Run 2	Run 3
I	2.5	1	2.5	2.5	2.6	1	1	1
II	2.4	1	2.1	2.1	2.1	1	1	1
III	2.3	1	2.1	2.1	2.1	1	1	1
IV	1.2	1	1.2	1.2	1.2	1	1	1
V	0.4	1	0.4	0.4	0.4	1	1	1
VI	3.6	1	3.6	3.7	3.7	1	1	1
VII	3.7	1.03	3.6	3.7	3.7	1	1	1
VIII	5.4	1.14	5.6	5.5	5.5	1.09	1.13	1.13
IX	1.6	1	1.5	1.5	1.6	1	1	1
X	1.6	1	1.6	1.6	1.6	1	1	1
XI	10.7	1.4	10.7	10.8	10.9	1.35	1.36	1.35
XII	3.8	1	3.9	3.9	3.9	1.04	1	1

design strategy to the in-vitro rapid screening program, a means of reducing the number of experiments, whilst retaining a broad search, was required. We present an answer to this problem by utilising the sensitivity and selectivity of mass spectrometric detection.

2. Experimental

2.1. Materials and reagents

Racemic 8-OH-DPAT was obtained from Aldrich (Poole, UK), and the analogues from Pharmacia and Upjohn (Kalamazoo, MI, USA). All solvents and reagents (HPLC or analytical reagent grade) used for chromatography were supplied by Fisons (Loughborough, UK). Water was prepared in-house using a Milli-Q water purification system (Millipore, Watford, UK).

2.2. HPLC instrumentation

The HPLC system consisted of a Hewlett Packard 1090 (Wokingham, UK) with the internal ultraviolet (UV) diode array detector set to 315 nm. The mobile phase flow rate used for all experimental procedures was 1 ml min^{-1} and the column oven temperature was set to 45°C.

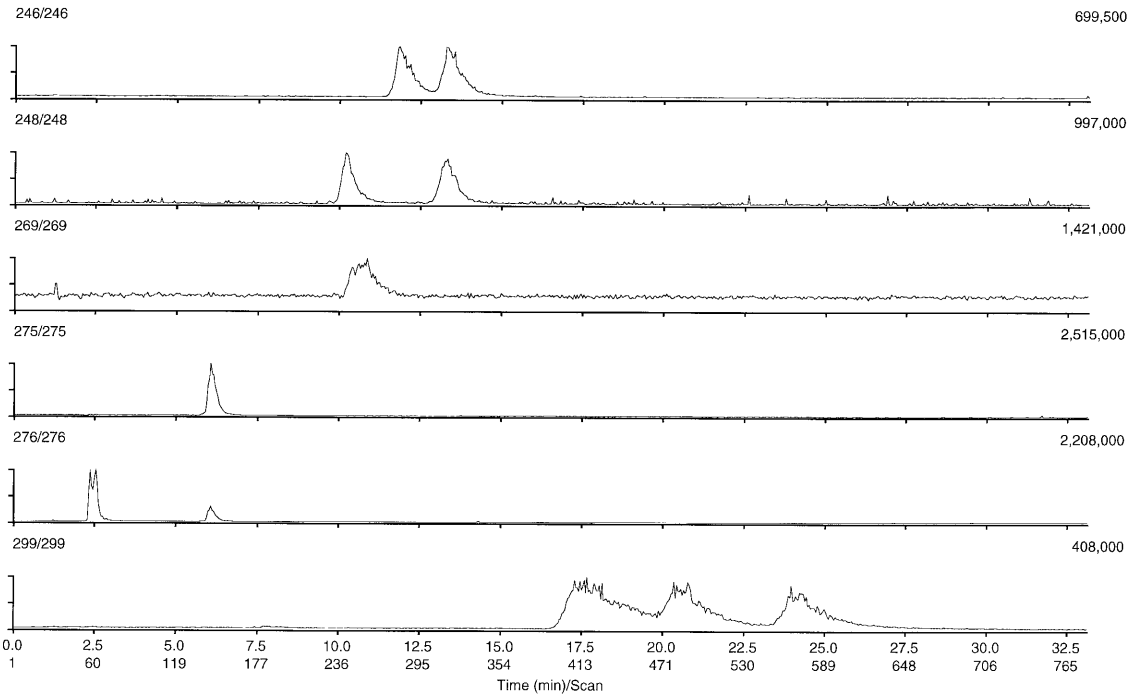

Fig. 2. Example of the SIM trace for compounds **I**–**VI**. Mobile phase composition no. 2 (mobile phase: ammonium acetate (0.05 M, pH 7.0):2-propranol (97:3 v/v)).

2.3. Mass spectrometry (MS)

Mass spectrometric detection was carried out using a Perkin–Elmer SCIEX API III$^+$ (Beconsfield, UK) in the SIM (single ion monitoring mode) using a heated nebuliser interface. The SCIEX was set to scan six discrete mass ranges (m/z 247.0, 267.0, 275.5, 299.0, 311.5 and 325.0) each ± 5 mass units wide. These ranges correspond to the mass range of the 12 analytes.

2.4. Chromatography

The chiral stationary phase used was Chiral AGP (150×4.6 mm) obtained from Hichrom (Reading, UK) and was used with various mobile phases as described in the text.

2.5. Factorial design experiments

A 2^3 factorial design experiment tested three mobile phase variables at two levels. These were pH (0.05 M, ammonium acetate), at either pH 4.0 or 7.0; hydrogen bonding capacity of the organic solvent modifier, either acetonitrile (low potential for H bonding) or 2-propanol; and concentration of organic solvent, either 3 or 8% v/v. For each of the eight experiments, the capacity factor (k') of the first eluting enantiomer and the relative retention (α) of the two enantiomers were recorded. The factor effects for these experiment were processed by the method of Yates [6].

3. Results

3.1. Chromatography

The eight mobile phase combinations required for the 2^3 factorial design are given in Table 1. The analogues represented a range of eight position substitutions with a reasonably consistent 2-aminotetralin substructure. Before conducting the factorial design, analyte interaction during

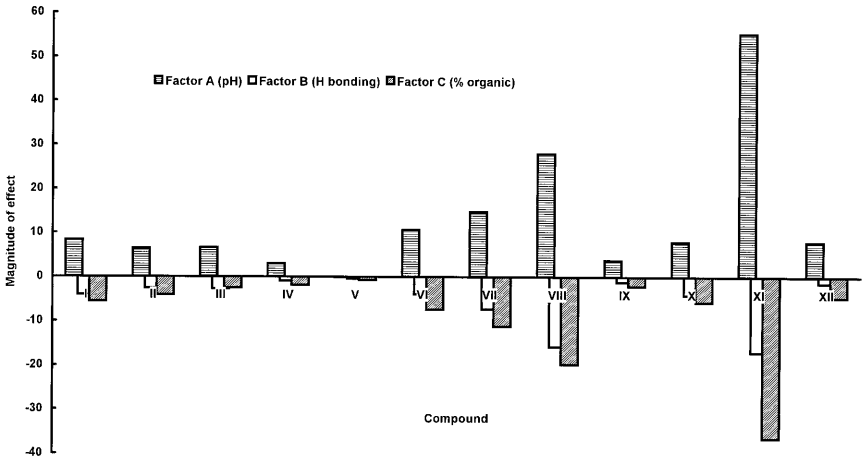

Fig. 3. Effect of factors upon the capacity factor recorded for the earlier eluting enantiomer of each analogue.

chromatography was addressed. Each analogue was injected separately (100 ng on column) and in combination with the other 11 analytes (8 ng each on column). The mixture was injected on three occasions and all injections were carried out in random order. Two compounds, **VI** and **VII**, had identical mass. They did, however, possess dissimilar UV absorbance profiles and consequently were resolved using an in-line UV detector. The results for k' and α for each compound are given in Table 2.

With the consistent total sample load, there appeared to be no interaction between the analytes in the mixture sample. Two low α values (1.04, **XII** and 1.03, **VII**) were recorded due to short-term noise at the apex of these peaks which was not seen in subsequent analyses. The eight factorial design experiments were run in random order, with an additional three using a mobile phase equivalent to the mid-point of the factorial space, i.e., ammonium acetate (0.05 M, pH 5.5):acetonitrile:2-propanol (94.4:2.25:2.25, v/v/v), to measure the degree of variability of the chromatography. The reproducibility of the chromatograms at the mid-point was similar to that seen for the repeat injections with mobile phase composition number 8, shown in Table 2. Examples of the single ion monitoring (SIM) chromatograms generated are shown in Fig. 2. The calculations were carried out using a simple PC spreadsheet program (Excel 6.0) and the resulting factor effects are summarised in Figs. 3 and 4.

The results obtained were consistent with those expected when using Chiral AGP. The positive factor effect for the pH of the mobile phase means that k' increased when the mobile phase pH increased from 4.0 to 7.0. The k' of compound **V**, a carboxylic acid substitution, did not increase with pH, giving a very small negative result for this factor effect. Increasing the organic solvent content of the mobile phase reduced the retention of all compounds with 2-propanol being more effective than acetonitrile.

It can be seen from Fig. 4 that four compounds did not resolve under any of the conditions tried. Of those analytes which were resolved, increasing the pH of the mobile phase always improved resolution. Only one compound, **I**, was better resolved with 2-propanol rather than acetonitrile. Table 3 summarises the chromatographic behaviour of each analyte.

4. Discussion

The object of the optimisation strategy was to enable the rapid development of enantioselective analytical methods for use with the isolated rat hepatocyte metabolic stability model. It is the use of LC MS that makes the experimental approach

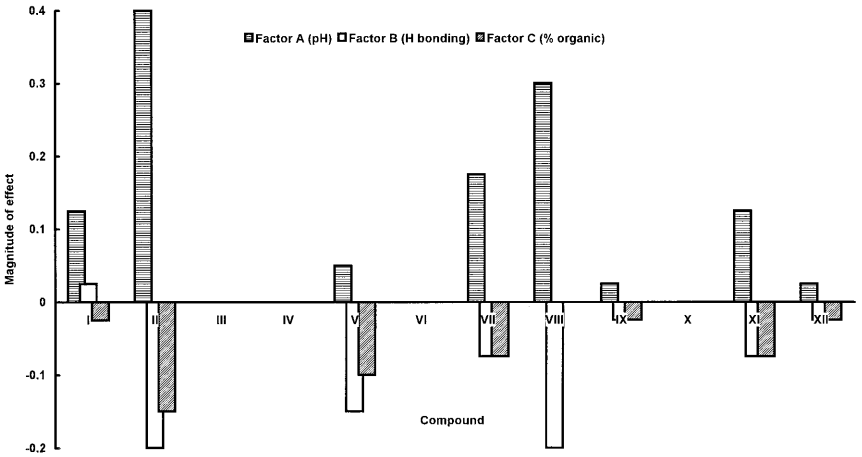

Fig. 4. Effect of factors upon the enantioselectivity recorded for each analogue.

possible. Similar experiments could be conducted with diode array UV detection, but the application would be limited to compounds with sufficiently different UV absorbance spectra. The number of analytes which could be tracked easily in a single analytical run would still be restricted. With LC MS, however, molecular ion differences in even close analogues are much more likely than sufficient differences in UV spectra.

The use of multiple scans of limited mass range (± 5 units) rather than scanning the whole range required by the molecular ions, resulted in a reduction in noise arising from the detection of unwanted masses. Due to the allowable increase in the dwell time (20 ms) of the mass spectrometer detector, an increase in sensitivity is also seen.

Some of the mobile phase combinations led to large capacity factors ($k' > 35$), and a shorter Chiral AGP column would have reduced the overall run time required to complete the factorial design. These long run times would not be necessary to produce adequate enantioselectivity and, if using the mass spectrometer, selectivity from the biological matrix. However, if UV detection was to be used subsequently for the analysis of in-vitro preparations, some of the separations obtained would not have been acceptable without further development of the chromatography. To achieve this, a number of options are possible, for example, the salt concentration of the mobile phase could have been increased. This has been shown previously to reduce retention but not enantioselectivity (for analyte **XI** [9]).

Data generated by these experiments can be used to improve the design of experiments for future analogues in the series. In this example, all of the separations obtained would have been possible using acetonitrile and a pH of 7.0. Exploration of the hydrogen bonding factor (factor B) could therefore be replaced by the exploration of a new factor, for example, the addition of a charged modifier such as dimethyloctylamine [10]. With sufficient data for a series of analogues, and different chiral stationary phases, it may be possible to include stationary phase as a factor in a single design, as long as both phases were compatible with the remaining two or more factors. The objective is to develop acceptable methods quickly, and under these circumstances factors without a linear relationship, as would be the case for different stationary phases, may be appropriate for inclusion into the design.

Those compounds not separated in the increasingly more focused factorial design can be examined separately with alternative experimental designs, chiral stationary phases or other stereoselective methods. It is important that these molecules that behave differently are quickly identified from the rest of the series.

Table 3
Mobile phase composition required to achieve resolution of enantiomers and the number of conditions under which a separation was obtained (maximum 8)

Compound	Comment	Best separation (PV[a])	No. of separations (PV ⩾ 0.2)
I	Separated only in presence of high pH	0.9	4
II	Separated in presence of high pH, not 2-propanol	1.0	4
III	No separation under all conditions	—	0
IV	No separation under all conditions	—	0
V	No separation using 2-propanol	1.0	3
VI	No separation under all conditions	—	0
VII	Separation with high pH but not with 2-propanol	1.0	3
VIII	Separation requires both high pH and CH_3CN	1.0	6
IX	One separation with high pH and low concentration of CH_3CN	0.9	1
X	No separation under all conditions	—	0
XI	Separation best with high pH, low % organic, particularly CH_3CN	1.0	8
XII	One separation with high pH and low concentration of CH_3CN	0.8	1

[a] Peak valley: the valley between two peaks as a fraction of the mean peak height (PV 1.0 = baseline resolution).

5. Conclusions

Under the conditions previously reported to achieve good resolution of **XI**, only one other analogue, **VIII**, was resolved on Chiral AGP. An additional six separations were achieved using only seven further chromatographic runs. The number of analytes that can be monitored simultaneously is limited by practical considerations. The 12 analytes used here produced 96 chromatograms from which the k' and α values were calculated by hand.

The predominant obstacle to the routine application of simultaneous factorial design is the requirement for sophisticated and, hence, expensive detection techniques, and perhaps the lack of software to allow the reliable calculation of k' and α automatically.

References

[1] C. Sonesson, M. Boije, K. Svensson, A. Ekman, A. Carlsson, A. Romero, I.J. Martain, J.N. Duncan, L.J. King, H. Wikstrom, J. Med. Chem. 36 (1993) 3409–3416.
[2] J.N. Duncan, T. Parton, T. Enos, Biochem. Soc. Trans. 18 (1990) 1200–1201.
[3] I.J. Martin, J.N. Duncan, A.H. Parton, W. Speed, L.J. King, Proceedings of the British Pharmaceutical Society, 1993, 163P–164P.
[4] S.A. Wood, S.A. Rees, R.J. Simmonds, J. Liq. Chromatogr. 20 (1991) 3761–3782.
[5] S.A. Rees, S.A. Wood, R.J. Simmonds, Methodological Surveys in Biochemistry and Analysis, Vol. 22, in: E. Reid, I.D. Wilson, (Eds.), The Royal Society of Chemistry, 1992, pp. 65–70.
[6] E. Morgan, Chemometrics: Experimental Design, John Wiley & Sons, 1991.
[7] R.P. Tucker, A.F. Fell, J.C. Berridge, M.W. Coleman, Chirality 4 (5) (1992) 316–322.
[8] J.O. DeBeer, C.V. Vandenbroucke, D.L. Massart, J. Pharm. and Biomed. Anal. 12 (11) (1994) 1379–1396.
[9] S.A. Wood, S.N. Pegg, R.J. Simmonds, D. Stevenson, J. Pharm. and Biomed. Anal. 14 (11) (1996) 1591–1599.
[10] J. Hermansson, Trends in Anal. Chem. 8 (7) (1989) 251–259.