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Development of chiral high performance liquid chromatographic assays for the enantiomers of 8-hydroxy-(di-*n*-propylamino)tetralin and three 8-keto-pyrrole-substituted analogues

S.A. Wood[a,*], S.N. Pegg[b], R.J. Simmonds[a], D. Stevenson[c]

[a]*Pharmacia & Upjohn Inc., Fleming Way, Crawley, West Sussex, RH10 3AL, UK*
[b]*School of Health Sciences, University of Sunderland, Sunderland, Tyne and Wear, SR1 3SD, UK*
[c]*Robens Institute of Health and Safety, Surrey University, Guildford, Surrey, GU2 5XH, UK*

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Abstract

Two chiral HPLC methods using protein-based stationary phases for the analysis of 8-hydroxy-(di-*n*-propylamino)tetralin and three 8-keto-pyrrole analogues are presented. Efficient solid-phase extraction enabled quantification of 0.02 μg ml^{-1} of the 8-keto-pyrrole analogues from 500 μl rat hepatocyte suspensions, and 0.2 μg ml^{-1} to be measured from 50 μl of rat plasma using UV detection at 315 nm.

Keywords: 2-Aminotetralin; Enantiomers; Factorial design

1. Introduction

Analogues of the 5-hydroxytryptamine (5-HT or serotonin) receptor agonist 8-hydroxy-(di-*n*-propylamino)tetralin (8-OH-DPAT) [1] (Fig. 1) were synthesised and screened for pharmacological activity in the search for novel, orally-active, CNS medicines. Active analogues were further screened for metabolic stability by an in-vitro model using isolated rat hepatocytes [2,3]. Relative stability data obtained from this model led to the preparation of new structures which, whilst retaining biological activity, had increased metabolic stability. The 2-aminotetralin substructure contains a chiral carbon atom (indicated by the asterisk in Fig. 1), but few of these compounds have been synthesised as pure enantiomers. Hepatocyte incubations were, for the most part, conducted on racemic mixtures with subsequent analysis using generic achiral chromatographic techniques [4,5], since these could be developed more quickly. Information regarding possible stereoselective metabolism of the 2-aminotetralin analogue enantiomers was not available using this approach, but the need to respond rapidly as part of the compound screening program was more important than the investi-

* Corresponding author. Present address: c/o 66 Westcott Crescent, Hanwell, London, W7 1PA.

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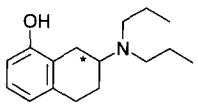
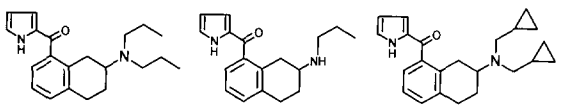


Fig. 1. 8-Hydroxy-(di-*n*-propylamino)tetralin.



I II III

Fig. 2. Keto-pyrrole-substituted analogues.

gation of chiral metabolism. If development of a chiral assay could be achieved with greater speed, then information regarding enantiomeric stability might assist the development of new analogues. Chiral assays were therefore explored further than the immediate needs of the project to investigate the possibility of developing a generic chiral assay for this class of compounds.

Two stereoselective HPLC methods are described, one for three 8-keto-pyrrole analogues of 8-OH-DPAT (Fig. 2) and a second method for 8-OH-DPAT itself, that have resulted from structure versus chromatographic selectivity experiments conducted on some of the 2-aminotetralins produced for the in-vitro screening program. The empirically derived mobile phase conditions for both columns were further tested using factorial design optimisation [6]. The first assay has been used to support in-vitro metabolism and pharmacokinetic studies with the 8-keto-pyrrole analogues [7].

2. Experimental

2.1. Materials and reagents

Racemic 8-OH-DPAT was obtained from Aldrich (Poole, UK), and the keto-pyrrole analogues **I**, **II** and **III** were supplied by Pharmacia & Upjohn Inc. (Kalamazoo, MI). All solvents and reagents (HPLC- or analytical-reagent grade) used for chromatography were supplied by Fisons (Loughborough, UK) with the exception of carobenzyloxy-glycyl-L-proline (ZGP) from Sigma (Poole, 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 Waters 600 multi solvent pump (Waters UK Ltd., Watford, UK), a Waters 712 WISP autosampler, a Shimadzu CTO-2A column oven (Dyson Instruments Ltd., Houghton-le-Spring, UK) and a Kratos Analytical Spectraflow 773 UV detector (Manchester, UK) operated at 315 nm for **I**, **II** and **III**, and 278 nm for 8-OH-DPAT. Unless otherwise stated the mobile phase flow rate used for all experimental procedures was 1 ml min^{-1} and the column oven temperature was set to 40°C. UV spectral analysis was carried out using a Waters 990 diode array detector. The ESA Analytical Coulochem instrument (Huntingdon, UK) provided electrochemical detection capability.

2.3. Mass spectrometry

Mass spectrometry used for confirmation of peak identity was carried out using a Finnigan MAT TSQ70 instrument fitted with a thermospray LC interface (Finnigan MAT, Hemel Hempstead, UK). LC–MS was investigated using a Perkin-Elmer SCIEX APCI (atmospheric pressure chemical ionisation) heated nebuliser with single ion monitoring.

2.4. Circular dichroism

The measurement of the optical rotation of the chromatographically isolated enantiomers was carried out using a Jasco J720 spectropolarimeter (Easton, MD). The dried HPLC fractions were redissolved in methanol and diluted to approximately 100 μg ml^{-1}. The solution was added to a 1 cm pathlength cuvette and scanned from 185–380 nm with a band width of 1 nm. Multiple (2–4) scans were made and averaged to reduce noise. Data were reported after baseline subtraction and normalisation.

2.5. Chromatography

Column screening was conducted using a number of commercially available chiral stationary phases (CSPs). Daicel Chiralcel OD (250 mm × 4.6 mm) and Chiralcel OJ (250 mm × 4.6 mm) columns from J.T. Baker (Milton Keynes, UK) were used with a Brownlee CN (15 mm × 3 mm) guard column from Perkin-Elmer (Beaconsfield, UK). The mobile phase consisted of 2-propanol–diethylamine–glacial acetic acid–hexane (25:0.1:0.1:74.8 v/v/v/v). The Astec β-cyclodextrin column Cyclobond 2000 I (250 mm × 4.6 mm) and an acetylated β-cyclodextrin column (250 mm × 4.6 mm) were obtained from BAS Technicol (Stockport, UK) and fitted with a Brownlee C2 (15 mm × 3 mm) guard column. For reversed-phase chromatography the mobile phase consisted of methanol–water–triethylamine (20:79.9:0.1 v/v/v). The water plus triethylamine was adjusted to pH 5.5 using glacial acetic acid prior to the addition of methanol. The column temperature used was 30°C.

The Cyclobond I 2000 column was also used with a non-aqueous polar solvent [8], acetonitrile–triethylamine–glacial acetic acid (99.7:0.1:0.2 v/v/v). The column temperature used was 5°C.

The protein-based columns Chiral AGP (150 mm × 4.6 mm) and Ultron ES-OVM (150 mm × 4.6 mm) were obtained from Hichrom (Reading, UK). Each column was used in conjunction with a Brownlee C2 (15 mm × 3 mm) guard column. The Chiral AGP column was used with 2-propanol–ammonium acetate (0.01 M, pH 5.0) (8:92 v/v) for **I** and **II**. For **III** the mobile phase used was 2-propanol–ammonium acetate (0.01 M, pH 6.7) (12:88 v/v).

For 8-OH-DPAT the mobile phase consisted of ammonium acetate (0.01 M, pH 6.7) or acetonitrile–ammonium acetate (0.01 M, pH 6.7) (15:85 v/v). The mobile phase used with the Ultron ES-OVM column for both 8-OH-DPAT and **II** was acetonitrile–potassium phosphate buffer (0.01 M, pH 7.0) (6:94 v/v).

The isolation of the enantiomers of 8-OH-DPAT was carried out using the method described by Ackland et al. [9]. The HPLC column used was a Lichrosorb DIOL (Hichrom), (250 mm × 4.6 mm). The mobile phase consisted of chloroform–triethylamine (500:0.166 v/v) plus 1.53 g of ZGP. The chromatography was conducted at ambient temperature and the flow rate was reduced to 1 ml min^{-1}.

2.6. Extraction of biological samples

Aliquots (100–500 μl) of calibration standards and study samples for the analysis of in-vitro incubates were prepared for extraction by protein precipitation with an equal volume of methanol. After centrifugation the supernatant was diluted with 1–2 ml of water and loaded onto primed (2 ml acetontirile followed by 2 ml water) Isolute C2 (EC) 100 mg, 3 ml, solid-phase extraction cartridges (Jones Chromatography, Hengoed, UK). Cartridges were rinsed with 1 ml of water followed by 1 ml of acetonitrile–water (30:70 v/v). After drying the cartridges by briefly applying a vacuum, analytes were eluted with 1 ml of acetonitrile–water–trifluroacetic acid (60:39.9:0.1 v/v/v). Eluates were dried using a centrifugal vacuum evaporator (GyroVap; G.A. Howe, Slough, UK), reconstituted with 100 μl mobile phase–water (50:50 v/v) and up to 80 μl was injected onto the column.

Aliquots of rat plasma (50 μl) were diluted with 2 ml of water before being extracted as for in-vitro incubates.

2.7. Calibration

Control rat hepatocyte suspensions were deactivated by heating to 50°C for 10 min and cooled prior to fortification with test substance. Calibration standards were prepared using deactivated hepatocyte suspensions in the range 0.02–20.00 μg ml^{-1}, and 500 μl aliquots were prepared for extraction. Calibration standards in rat plasma (potassium EDTA anticoagulant) were prepared in the range 0.2–20.00 μg ml^{-1} and 50 μl aliquots were extracted.

2.8. Factorial design experiments

A 2^3 factorial design experiment was conducted with the Chiral AGP CSP column. Three mobile

phase variables (factors) were investigated: pH (5.44 and 7.00), percentage of 2-propanol (8 and 12) and sodium phosphate buffer molarity (0.005 M and 0.05 M). For each of the eight experiments the retention of the first eluting enantiomer was recorded with the relative retention (α) and the peak valley ratio [10] (PV) of the two enantiomers. The factor effects for this experiment were proposed by hand.

A further experiment was conducted using the Ultron ES-OVM CSP column. A 2^3 star design was superimposed upon a 2^3 factorial design. The composite factorial design [11] investigated three mobile phase factors: acetonitrile concentration (2, 6, 14 and 18%), potassium phosphate buffer concentration (0.005 M, 0.01 M, 0.1 M and 0.25 M) and pH of the buffer (3, 4, 6 and 7). This resulted in 14 experiments. An additional point was added at the centre of the factorial space: 10% acetonitrile, pH 6 and 0.05 M buffer. The chromatographic data collected from the experiment were analysed using the statistical software program SAS (SAS/Windows V6.08, SAS Institute Inc., NC, USA). The regression equations fitted to the data were used to produce three-dimensional response surface plots showing the interaction between any two factors.

3. Results and discussion

3.1. Chromatography

Partial separations were achieved for **I**, **II** and **III** using the 3,5-dimethylphenylcarbamate-derivatised cellulose column, Chiralcel OD, but the chromatographic performance was poor and the complete resolution of the enantiomers could not be achieved without peak broadening and tailing leading to lower sensitivity. Substituting a Chiralcel OJ column resulted in loss of selectivity, suggesting involvement of the amide nitrogen in either a dipole or hydrogen bonding interaction. No resolution of the 8-OH-DPAT enantiomers was found with either column. Due to the preference for direct compatibility with mass spectrometry, i.e. an aqueous mobile phase, no further work was carried out using these stationary phases. The reversed-phase Chiralcel OD-R column was not available for testing.

No separations of **I**, **II**, **III** or 8-OH-DPAT using cyclodextrin-based CSPs were found. Given the separations achieved with Chiralcel OD it was unfortunate that the cyclodextrin derivatised with 3,5-dimethylphenylcarbamate (Cyclobond DMP) was not tried. Considering effort was spent investigating a reversed-phase separation using β-cyclodextrin. The resolved peak was eventually found to be a system peak related to the triethylamine concentration of the mobile phase.

Chiral AGP resolved the enantiomers of **I**, **II** and **III** using a mobile phase of sodium phosphate buffer (0.1 M, pH 5.0). The retention and stereoselectivity observed for each of the analogues increased with increasing mobile phase pH. Retention was reduced by the addition of 2-propanol. Acetonitrile was tried as an alternative modifier but did not improve resolution. Increasing the column temperature, to 40°C, reduced the retention of the enantiomers but not the selectivity of the column, and the elevated column temperature was retained to reduce the mobile phase viscosity and the effect of temperature variation in the laboratory. Two partial separations of the 8-OH-DPAT enantiomers were obtained using Chiral AGP CSP although neither separation was adequate to support in-vitro studies.

The 2^3 factorial design experiment was conducted to ascertain the relative importance of the factors shown to affect the steoreoselectivity of the Chiral AGP column. The more polar analogue, **II**, was chosen for the experiment. The best separation obtained is shown in Fig. 3 and relative effects of the three factors on the measured parameters are given in Table 1. The results of the factorial experiment confirm that increasing mobile phase pH resulted in greater retention and stereoselectivity, and increasing the concentration of 2-propanol has the opposite effect. Increasing the molarity of the mobile phase buffer significantly reduced retention but did not affect stereoselectivity. The data suggest a hyrophobic binding site with an additional ionic interaction (via the basic nitrogen of the analyte) which is important for binding but not directly involved in the stereoselectivity. Chromatography and molec-

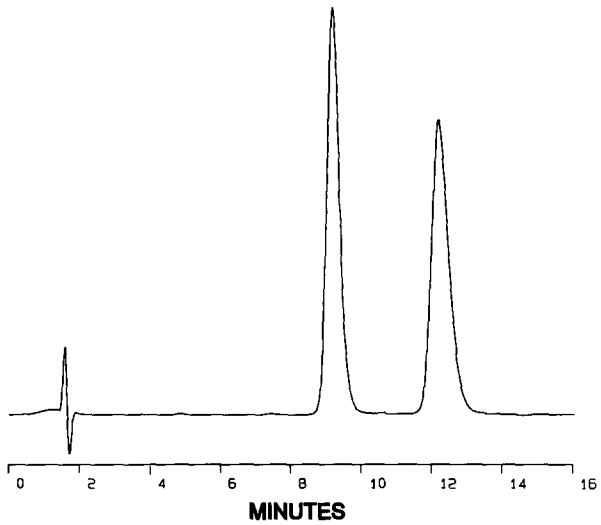


Fig. 3. Example chromatogram for **II** using Chiral AGP (sodium phosphate buffer (pH 7.00, 0.005 M)–2-propanol (92:8 v/v)).

ular modelling experiments, by the authors, with other 2-aminotetralin analogues and Chiral AGP (presented in poster format at the 3rd Int. Symp. on Chiral Discrimination, Tubingen, Germany, 1992) indicated that a hydrogen accepting group attached to the 8 position was required for chiral recognition.

A separation for 8-OH-DPAT has been previously reported using both Chiral AGP and Ultron ES-OVM [12]. Using the Ultron ES-OVM column the enantiomers of 8-OH-DPAT and the analogues were well resolved with a mobile phase pH of > 5. A further factorial design experiment, with the addition of a superimposed star design, was carried out using 8-OH-DPAT and **II**. The experiments were conducted in random order.

The two points of the 2^3 factorial design experiment used with the Chiral AGP CSP column assume a linear relationship between the factors and the measured response; the additional data points of the composite design allow a non-linear relationship to be approximated. The retention and selectivity data obtained from the Ultron ES-OVM column were, however, best approximated using a linear fit. The relative importance of the mobile phase factors and their statistical significance are shown in Table 2.

The interaction of pH and acetonitrile concentration was the most significant upon retention and separation of both analytes, but the values produced for the factor interactions are difficult to interpret [6]. Considering only the individual factors, the data show that increasing the pH of the

Table 1
Relative effects of mobile phase factors on chromatographic performance of **II** calculated from the results of the 2^3 factorial experiment using Chiral AGP

Factor	K'	α	PV
pH of mobile phase	6.22	0.29	0.06
% 2-Propanol in mobile phase	−2.44	−0.30	−0.07
Molarity of mobile phase buffer	−4.13	0.06	−0.02

Table 2
Relative effects of the three factors investigated upon the retention and stereoselectivity of the Ultron ES-OVM CSP column for **II** and 8-OH-DPAT

Factor	**II**		8-OH-DPAT	
	K'	α	K'	α
% Acetonitrile	−2.43[a]	−0.13	−1.48	−0.18
Mobile phase pH	1.88[a]	0.22[a]	1.35[a]	0.32[b]
Buffer molarity	−0.30	−0.01	−0.11	−0.08

[a] Value is significant at the 5% level.
[b] Value is significant at 10% level. The remaining values were not significant.

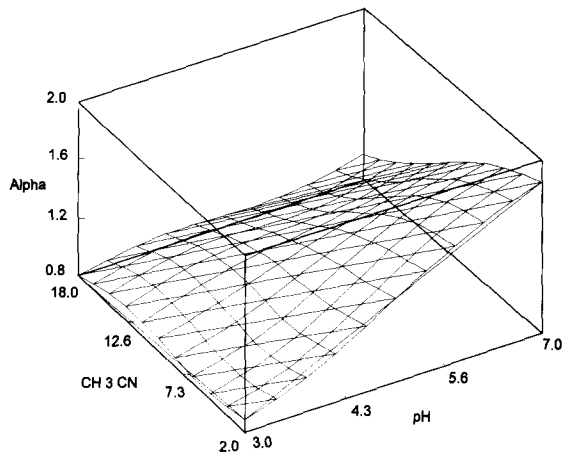


Fig. 4. Surface response plot for the effects of mobile phase pH and acetonitrile concentration upon the relative retention (α) of the enantiomers of 8-OH-DPAT.

mobile phase resulted in significant increases in retention and selectivity. The acetonitrile concentration of the mobile phase was not as important when considered in isolation. The effect of buffer concentration in the range investigated was not significant. Fig. 4 shows the surface response plot generated from the factorial design data where the effect of acetonitrile concentration can be seen to be important only at high pH. Figs. 5a and 5b show the best separation achieved for 8-OH-DPAT and **II** respectively.

3.2. Confirmation of enantiomeric separation

Enantiomerically pure standards of the compounds under investigation were not available. UV spectral analysis using diode array detection resulted in identical UV spectra over the range 200–450 nm for each of the presumed enantiomer pairs of the analogues. These peaks were further examined using mass spectrometry. Both peak pairs exhibited the same molecular ions and fragmentation pattern. To confirm optical activity, the enntiomers were isolated in semi-preparative runs using the Chiral AGP column (column loading 30 μg per injection) and circular dichroism (CD) spectra were taken (Fig. 6). An attempt was made to assign the configuration of the enantiomers by comparison with the CD spectra of 8-OH-DPAT enantiomers. The enantiomers of 8-OH-DPAT were prepared using a published method [9] where the elution order of the *R* and *S* enantiomers had been determined. However, the CD spectrum in the region of interest (190–225 nm) was too dissimilar to that of the analogues for an assignment to be made. It was, however, possible to conclude that the elution order of the enantiomers of **I**, **II** and **III** was the same when using Chiral AGP.

3.3. Extraction development

Biological samples from the in-vitro screening program required, at most, only protein precipitation for achiral analysis. The Zorbax RX C8 columns used provided sufficient selectivity and robustness. However, this method of sample preparation was not adequate for either of the less robust protein-based CSPs.

Extraction of **II** and **III** from a biomatrix was tried with a range of bonded-phase solid-phase extraction (SPE) cartridges: C8, PH, Cyclohexyl, C2, CN, 20H and CBA. Complete recovery of both enantiomers was obtained from each cartridge using an elution solution of acetonitrile–water–trifluroacetic acid (60:39.9:0.1 v/v/v). The C2 and CBA cartridges could be rinsed with up to 50% methanol in water without loss of analyte. The acidic elution solvent caused baseline disturbances with Chiral AGP and a centrifugal vacuum evaporator (GyroVap) was used to completely remove the volatile trifluroacetic acid prior to redissolving the extracts for injection. Both 8-OH DPAT and **I** was efficiently extracted with C2 and CBA cartridges whilst much of the endogenous biological matrix was eliminated. The C2 cartridge was chosen for subsequent analytical development because it offered a degree of mode sequence relative to the analytical column [13] Chiral AGP.

The recovery, using C2, was $>92\%$ (at 2 μg ml^{-1}, RSD = 2.1, $n = 5$) for both **II** and **III** from control rat hepatocytes and $>93\%$ (at 2 μg ml^{-1}, RSD = 2.5, $n = 5$) from control rat plasma. Chromatograms were free from interfering endogenous peaks due to the efficient extraction and in part to the selective wavelength used for detection, 315 nm.

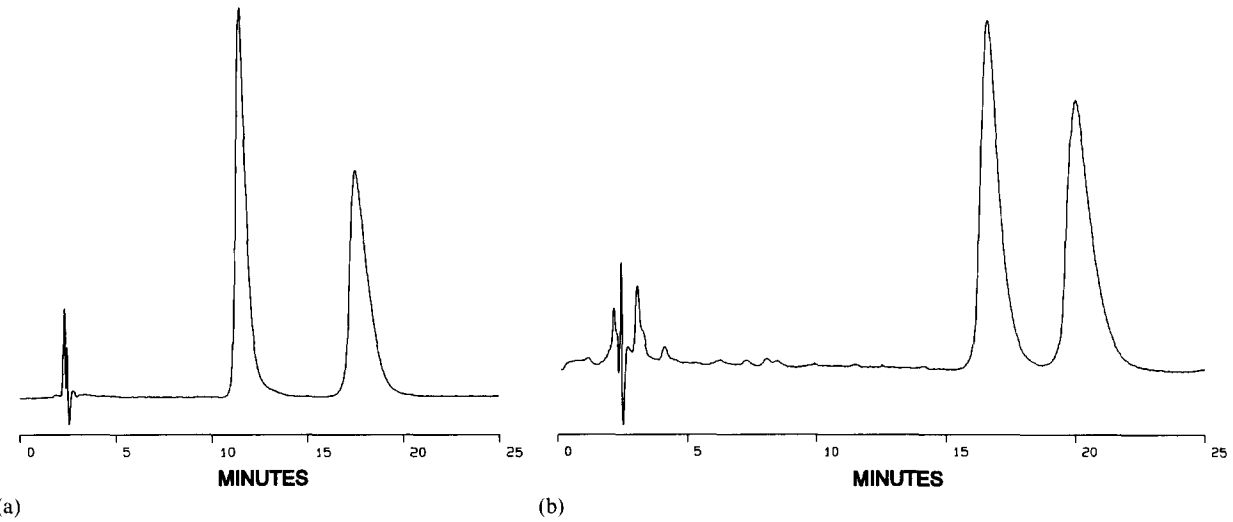


Fig. 5. Example chromatograms for (a) 8-OH-DPAT and (b) I using Ultron ES-OVM (acetonitrile–potassium phosphate buffer (pH 6.0, 0.1 M)(6:94 v/v)).

3.4. Calibration

The achiral analysis of the in-vitro metabolism incubates was conducted using a single external standard only. No loss of analyte was seen during the protein precipitation procedure and the assay relied upon the accuracy and precision of the sampling of the incubate, the addition of the methanol used to arrest the metabolism, and the precision of the autosampler. The chiral method required more sophisticated extraction which introduced a possible source of variation. The use of an internal standard was, however, not possible because of the difficulty in finding suitable, preferably enantiomericaly pure, candidates and the limited peak capacity of the protein CSPs.

Calibration curves for incubates and plasma were linear ($r^2 > 0.98$, $n = 14$) using a weighted ($1/x^2$) regression. After demonstrating that the extraction procedure was efficient and invariable for all the concentrations, subsequent analysis of sample incubates was carried out using external standardisation only.

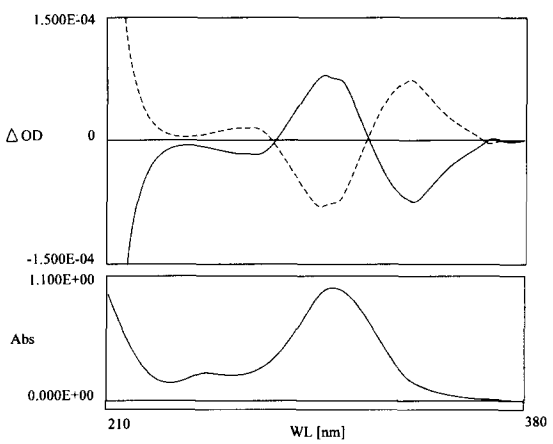


Fig. 6. CD spectra for the isolated enantiomers of I.

3.5. Sensitivity enhancement

A number of experiments were conducted to improve upon the sensitivity of the UV assay. Electrochemical detection (ESA Coulachem, + 0.7 V) was approximately 10 times more sensitive than UV. However, the baseline stability of the assay was poor and the major metabolite of the analogues, the *N*-despropyl, was not detected by this method. A method of improving the sensitivity of the UV detection by application of a short salt concentration gradient (0.01–0.1 M ammonium acetate in 20 min) whilst keeping both the pH (6.5) and the 2-propanol concentration (12%) constant resulted in peak heights approximately double the non-gradient response for es-

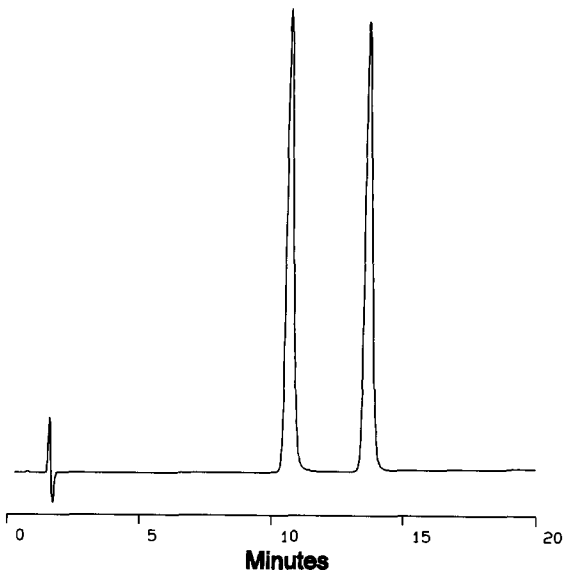


Fig. 7. Analogue **II** on Chiral AGP using gradient elution: solvent A = ammonium acetate (pH 6.5, 0.01 M)–2-propanol (88:12 v/v); solvent B = ammonium acetate (pH 6.5, 0.1 M)–2-propanol (88:12 v/v). Gradient slope = 100% A to 100% B in 20 min.

sentially similar retention times, Fig. 7. The sharper chromatographic peak also led to improved integration (Waters 860) for low concentrations of analyte. However, due to the lack of reliable gradient pumping systems gradient elution was not used routinely.

Atmospheric ionisation mass spectrometry detection using single ion monitoring was also an order of magnitude more sensitive than the isocratic UV assay. Daughter ion monitoring would potentially have shown greater sensitivity but the necessary resource was not available.

3.6. Application

The Chiral AGP assay has been used extensively for the measurement of samples generated by in-vitro incubation studies using **I** and **III**. The chromatography was adequate to resolve a number of metabolites found in vitro (Fig. 8). The Chiral AGP column was robust enough for the injection of 40–50 extracts before significant degradation of the chromatography was seen. Replacement of the guard column and flushing with 2-propanol–water (50:50, v/v) was adequate for regeneration of the chromatograph.

4. Conclusion

Effective chiral separations were achieved for the analysis of the 2-aminotetralin analogues **I**, **II** and **III** and for 8-OH-DPAT. The two factorial design experiments were conducted to see if better optimisation of the two assays was possible by this empirical approach which may then have lead to broader applicability to other analogues in this class. However, the drawback of factorial design strategies to support discovery programs is that they are not rapid enough. The number of experiments required is very large if a significant factor space is explored with a number of compounds.

Whilst these experiments gave insight into the way that the mobile phase constituents influenced separation, they did not lead to separations better than had previously been found. Nor did they lead to the prospect of a generic chiral method for these compounds. The application of the facotrial design approach to these assays was basic and much more thorough application of the data produced is possible [14,15].

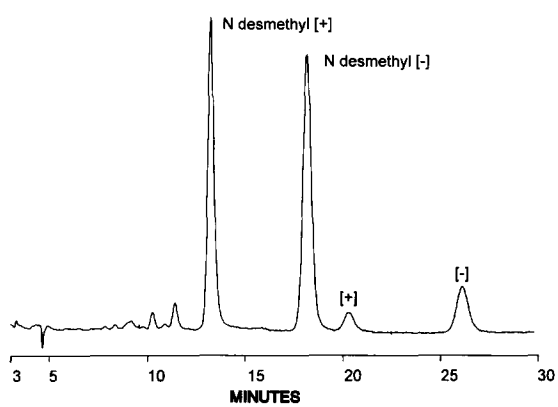


Fig. 8. Example chromatogram of extracted hepatocyte incubation medium after 20 min incubation with **III**. Optical rotation measured at 315 nm in methanol.

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