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Chiral resolution of protein kinase C inhibitors by reversed-phase high-performance liquid chromatography on cellulose tris-3,5-dimethylphenylcarbamate

J.A. Whatley

Physical Methods Department, Roche Research Centre, Roche Products Ltd., Welwyn Garden City, Herts. AL7 3AY, UK

Abstract

Protein kinase C (PKC) inhibitors are under investigation as potential anti-arthritic drugs. Attempts to resolve a lead compound into its respective enantiomers were made using normal chiral phase methodologies, e.g. Chiralcel OD, OG, OJ using isopropanol–hexane mixtures. Although good retention was achieved on some chiral columns, no separation was seen.

Silica-based cellulose tris-3,5-dimethylphenylcarbamate, Chiralcel OD, has been used previously to successfully resolve various drugs by reversed-phase HPLC using aqueous buffer–acetonitrile eluting systems.

This work investigates the direct chiral resolution of a range of PKC inhibitor racemates by reversed-phase chiral HPLC on Chiralcel OD using acetonitrile–phosphate buffer mixtures. The effect on the separation was studied with variables such as acetonitrile concentration, flow-rate, buffer pH and column temperature.

The effect on chromatography at sub-ambient temperature is discussed and examples are given where enhancement on resolution was achieved. In some cases baseline separation was obtained allowing determination of enantiomeric excess down to 0.1% level.

Although an increase in band broadening was observed as the temperature was reduced it did not reach an unacceptable level, even at 0°C.

1. Introduction

Separation of enantiomers by chiral HPLC is now well established with over 50 different chiral phases commercially available. Cellulose and amylose derivatives coated onto a silica backbone have been used extensively for chiral resolution using normal-phase conditions. Protein kinase C (PKC) inhibitors are under investigation as potential anti-arthritic drugs [1,2]. Modelling studies suggested that the individual enantiomers of bisindolylmaleimides such as Ro 31-8830 should have different activities against the enzyme [3].

Attempts to resolve Ro 31-8830 into its respective enantiomers, Ro 32-0432 and Ro 32-0434 were made using normal chiral phase methodologies, e.g. Chiralcel OD, OG, OJ and Pirkle phases with isopropanol–hexane mixtures. Although good retention was achieved on some chiral columns, no separation was seen. Silica-based cellulose tris-3,5-dimethylphenylcarbamate (CDMPC), Chiralcel OD-R, has been used previously to successfully resolve various drugs by reversed-phase HPLC using aqueous buffer–acetonitrile eluting systems [4,5].

This report investigates the direct chiral resolution of Ro 31-8830 and other PKC inhibitor

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racemates by reversed-phase chiral HPLC on Chiralcel OD-R. The effect on separation was studied with variables such as acetonitrile concentration, flow-rate, buffer pH and column temperature.

2. Experimental

Chiral chromatography was carried out on a Chiralcel OD-R column, Daicel (250 × 4.6 mm I.D.) using a Kontron 400 series liquid chromatograph equipped with a dual-wavelength detector operating at wavelengths 215 and 254 nm. Chromatographic data were collected and processed on a Kontron data system 450.

HPLC-grade acetonitrile was obtained from Rathburn. Water used to prepare the buffer was purified by a Milli-Q system. The buffer, triethylammonium phosphate, was prepared by adding triethylamine (Fluka) to 0.05 *M* phosphoric acid (Fluka) until the desired pH was reached. Adjustment of pH was made before addition of the organic modifier.

Compounds were chromatographed by dissolving them in the mobile phase at a concentration of 1 mg/ml. They were filtered through a 2-μm acro-disc and 10-μl injections were made via the auto sampler. A column chiller purchased from Jones Chromatography was used for experiments carried out at sub-ambient temperature.

3. Results and discussion

The structures of the PKC racemic compounds studied in this work are listed in Fig 1.

The results of the chiral separation of the twenty racemates chromatographed at 20°C are summarised in Table 1. The compounds were eluted with acetonitrile–phosphate buffer pH 2.5 (25:75) at a flow-rate of 0.5 ml min^{-1}, where $\alpha \geq 1.20$: baseline separation; $\alpha = 1.15$: near-baseline separation; and $\alpha = 1.05$: enantiomers just separate.

Fig. 2 is an example typical of the separations achieved for the majority of the compounds under test.

3.1. Chromatography at sub-ambient temperature

Retention and selectivity typically are adjusted by modifying the mobile phase composition or by changing the chromatographic column used. Chromatography at sub-ambient temperatures can reduce column efficiency due to poor mass transfer, arising from increased eluent viscosity and decreased solute diffusivity. The use of column cooling in chiral HPLC can be a valuable technique for improving column selectivity for racemates. This report highlights the benefits of sub-ambient temperature to enhance the resolution of racemates. The PKC inhibitor racemates Ro 31-8830 and Ro 31-8425 were used as typical examples to illustrate this effect. Chromatograms for both compounds were run at 5°C intervals from 20 to 0°C. Before each temperature study the column was conditioned for 30 min with mobile phase flowing at 0.5 ml min^{-1}. A plot of retention factor (α) against column temperature was made (Fig. 3). A near-linear relationship was obtained in which baseline separation is achieved at 0°C. The chromatogram of racemate Ro 31-8830 at 0°C (Fig. 4) shows the baseline separation achieved between the enantiomers Ro 32-0432 and Ro 32-0434. This method was used to determine the presence of the unwanted enantiomer Ro 32-0434 in various large-scale samples of Ro 32-0432. Detection down to 0.1% level was possible (Fig. 5).

Good thermal contact between the column and the base of the chiller ensured an even temperature throughout the length of the column. Three thermocouples placed at the beginning, middle and end of the column showed virtually no difference in the set temperature. An appreciable pressure increase was observed as the column was cooled to 0°C, but at the experimental flow-rate of 0.5 ml min^{-1} using acetonitrile–phosphate buffer (25:75) the back pressure did not exceed 2500 p.s.i. (1 p.s.i. = 6894.76 Pa), which therefore was acceptable.

The effect of increasing the pH of the phosphate buffer to 4.0 caused an increase in retention time with little overall effect on the separation. The pK_a of the ionisable amino

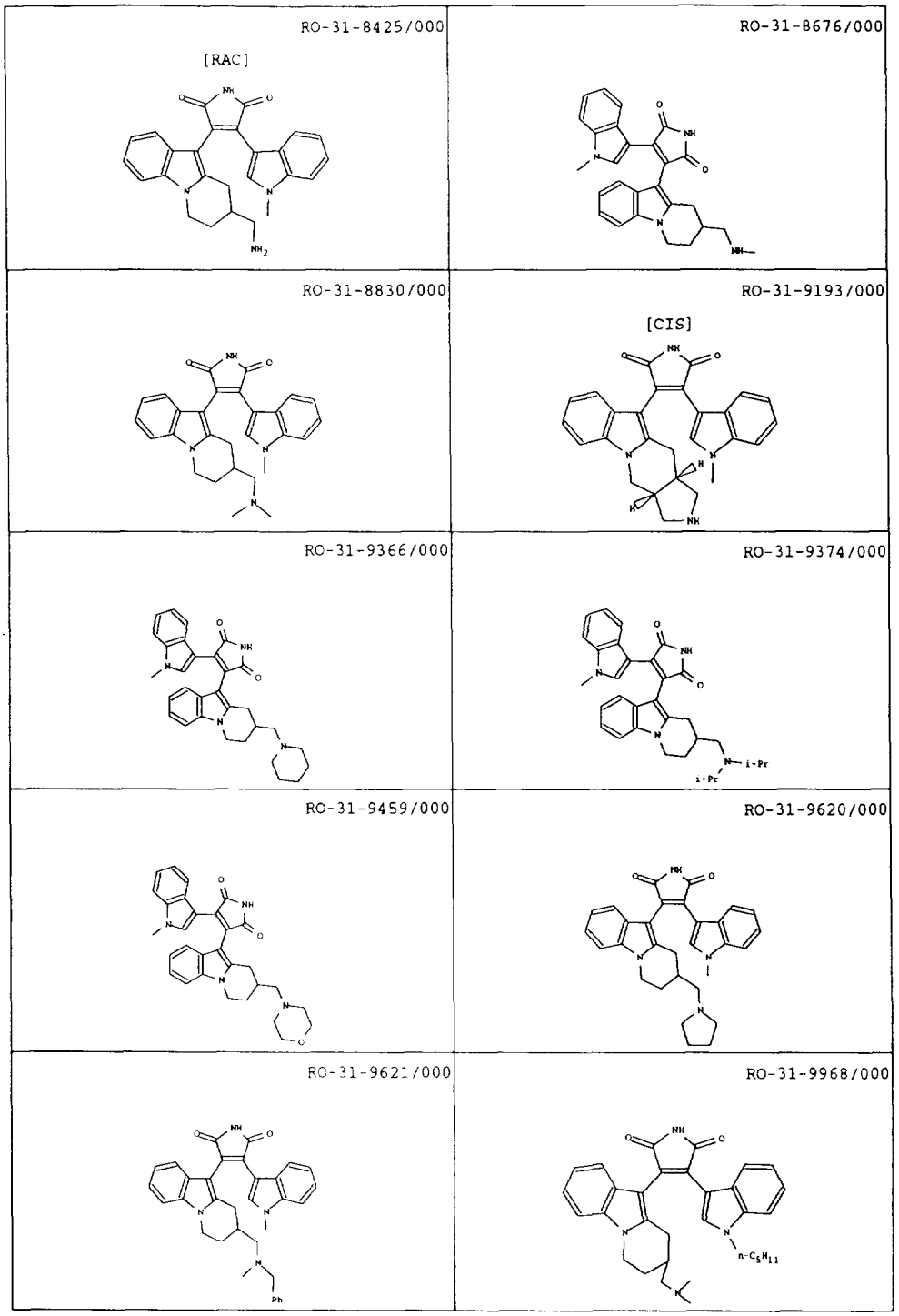

Fig. 1 (*continued on p. 266*).

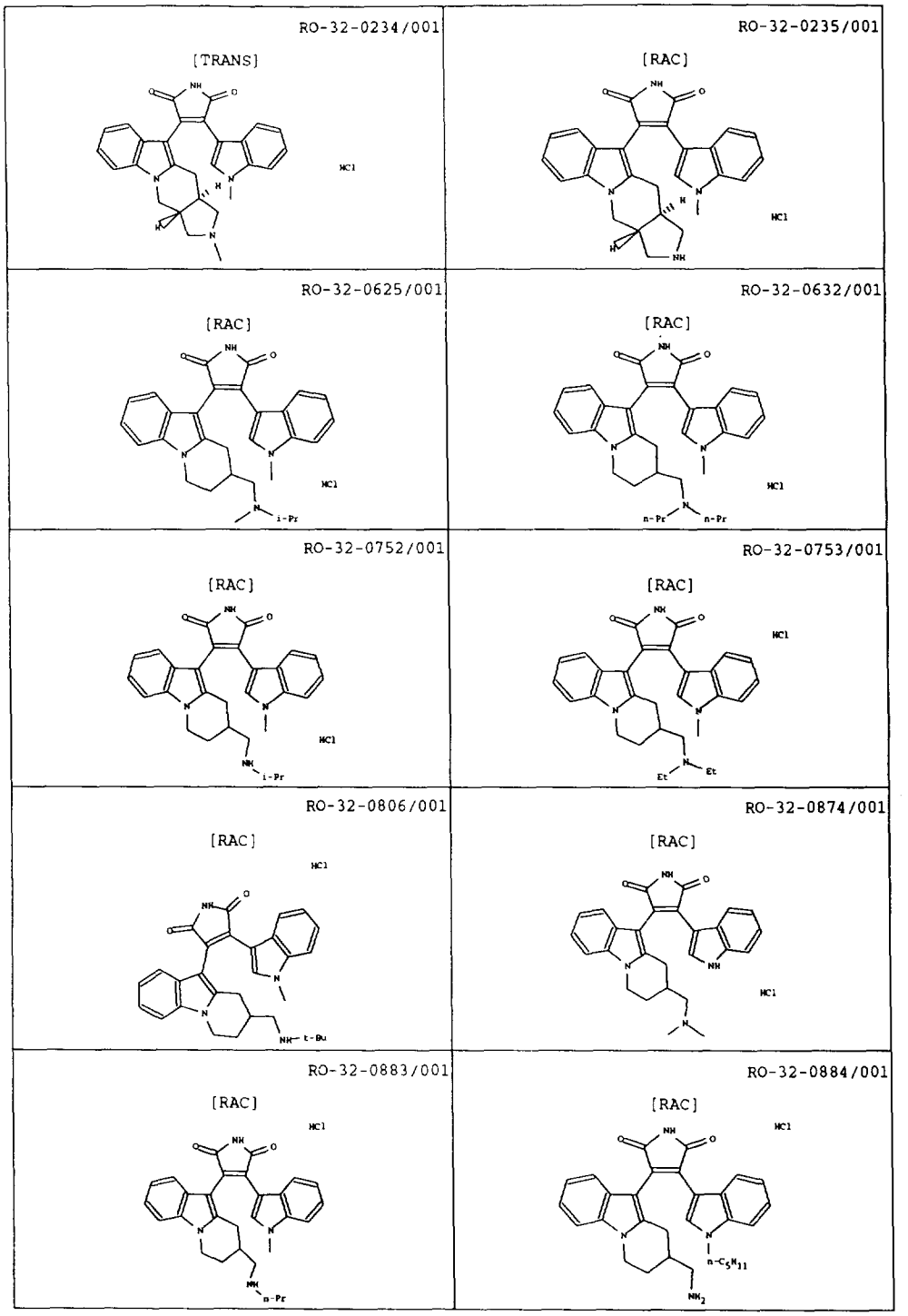

Fig. 1. Structures of racemic compounds under investigation.

Table 1
Comparison of retention of twenty chiral PKC inhibitors

PKC racemate	k'_1	k'_2	α
Ro 31-8830	4.34	4.90	1.13
Ro 31-8425	3.64	4.20	1.15
Ro 31-9366	8.44	9.76	1.16
Ro 31-9459	6.34	7.48	1.18
Ro 31-9620	6.50	7.36	1.13
Ro 31-8676	4.78	5.74	1.20
Ro 31-9193	4.42	4.58	1.04
Ro 31-9374	12.28	15.72	1.28
Ro 31-9621	26.62	29.82	1.12
Ro 31-9968	31.54	33.80	1.07
Ro 32-0625	7.54	8.92	1.18
Ro 32-0753	7.16	8.26	1.15
Ro 32-0874	2.94	3.12	1.06
Ro 32-0234	4.56	5.84	1.28
Ro 32-0235	3.58	4.58	1.28
Ro 32-0632	21.40	25.2	1.18
Ro 32-0752	6.40	8.10	1.27
Ro 32-0806	7.80	10.54	1.35
Ro 32-0883	7.36	9.58	1.30
Ro 32-0884	27.02	28.28	1.05

k'_1 = Retention factor of first-eluting enantiomer; k'_2 = retention factor of second-eluting enantiomer; α = retention factor.

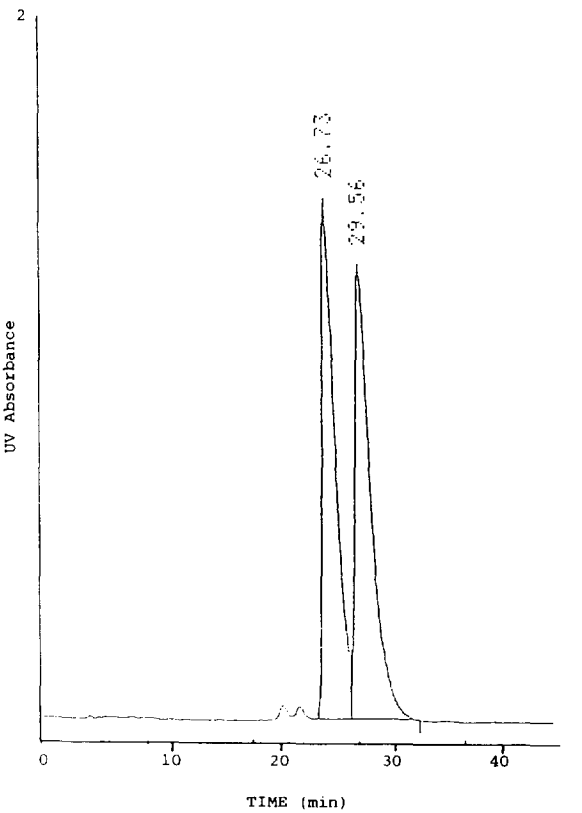

Fig. 2. Optical resolution of Ro 31-8830. HPLC conditions: Chiralcel OD-R; CH_3CN–phosphate buffer (25:75), pH 2.5; 0.5 ml min^{-1}; UV detection at 254 nm; 20°C.

group in the compounds under investigation is approximately 10. Increasing the acetonitrile concentration above 25% decreased the retention time with subsequent loss of resolution. Reducing the organic modifier below 25% increased the retention time with subsequent loss of peak shape and poor efficiency.

Following the success with Chiralcel OD-R in separating PKC inhibitors other chiral phases of similar chemical composition were tried using reversed-phase methodology. Chiral phases OG (cellulose tris-4-methylphenylcarbamate), OC (cellulose tris-phenylcarbamate) and AD (amylose tris-3,5-dimethylphenylcarbamate) were tested as possible candidates. Racemate Ro 31-8830 was used as the test compound. The OG phase showed some chiral recognition but only produced a slight separation. Chiral phase AD retained the racemate on the column without effecting a separation. The pressure on the OC column increased significantly as the mobile phase was passed through the column (acetonitrile–phosphate buffer, 25:75) at 0.5 ml min^{-1} and blockage occurred very quickly.

4. Conclusions

Chiralcel OD-R used in reversed-phase mode has proved to be a useful separation technique for resolving racemic PKC inhibitors. Chromatography at reduced temperature, down to 0°C, has proved effective in separating racemates that are difficult to separate at ambient temperature. Although an increase in band broadening was observed as the column temperature was reduced it did not reach an unacceptable level, even at 0°C.

Low-temperature chiral HPLC has been particularly useful in determining enantiomeric ex-

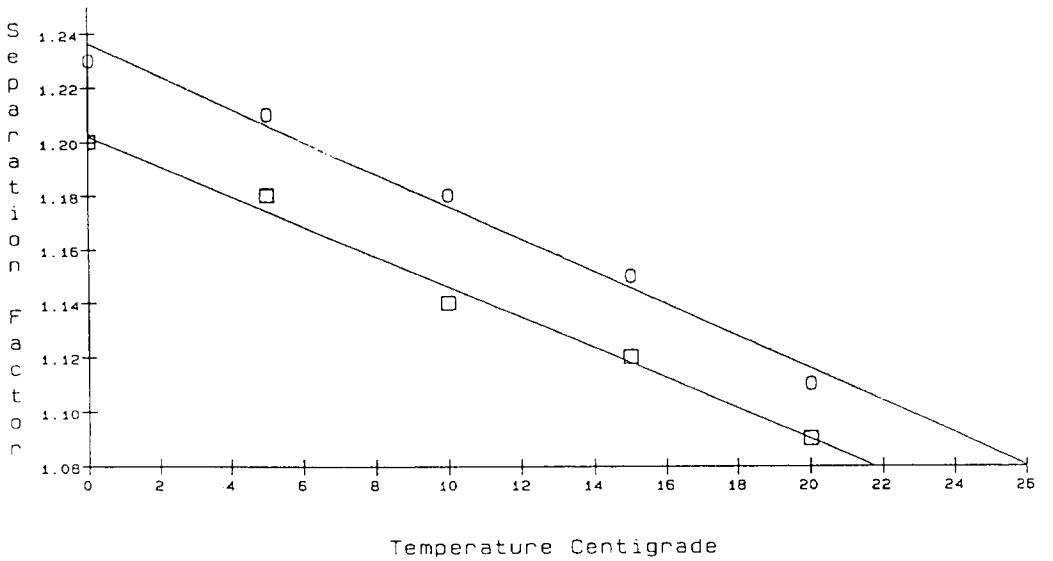

Fig. 3. PKC inhibitors Ro 31-8425 (□) and Ro 31-8830 (○): plot of separation factor vs. column temperature.

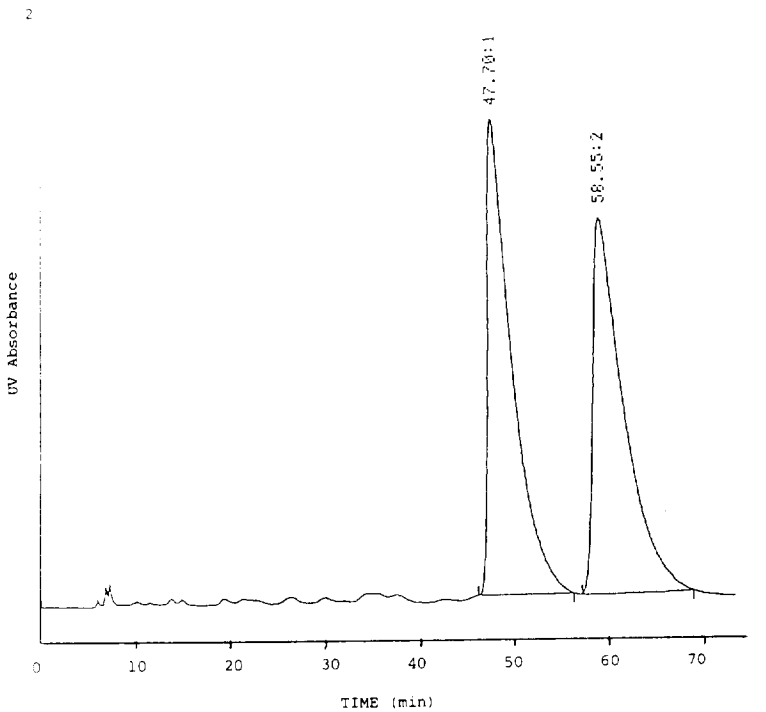

Fig. 4. Optical resolution of Ro 31-8830 at 0°C (α = 1.23). HPLC conditions: Chiralcel OD-R; CH_3CN–phosphate buffer (25:75), pH 2.5; 0.5 ml min^{-1}; UV detection at 254 nm; 20°C.

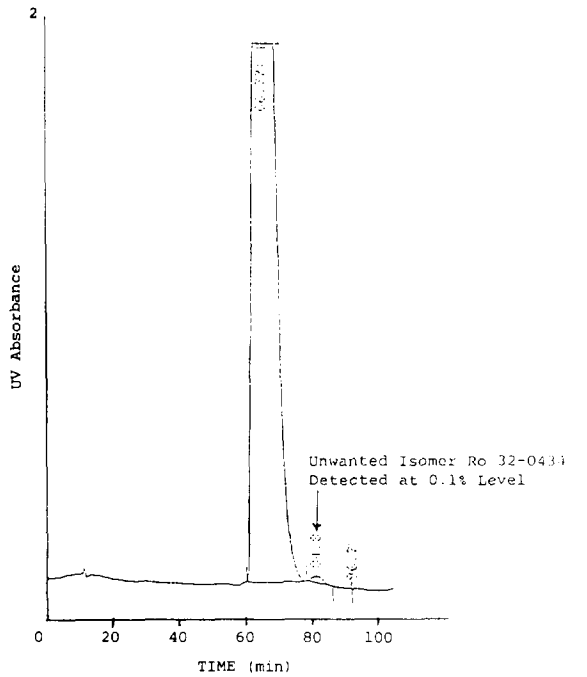

Fig. 5. Determination of the optical purity of Ro 32-0432 down to 0.1% level of unwanted enantiomer, Ro 32-0434. HPLC conditions: Chiralcel OD-R; CH_3CN–phosphate buffer (25:75), pH 2.5; flow-rate reduced to 0.3 ml min^{-1} for improved separation; UV detection at 254 nm; column temperature 0°C.

cesses of racemic compounds at low levels of detection. During this study the methodology was used to determine the presence of the unwanted enantiomer Ro 32-0434 in Ro 32-0432 down to the 0.1% level. The desired enantiomer Ro 32-0432, *S* configuration, eluted first from the column. The IC_{50} vs. PKC was 19 n*M* for the *S*-enantiomer and 90 n*M* for the *R*-enantiomer (Ro 32-0434). Substituting 0.1% trifluoroacetic acid for phosphate buffer would allow small-scale preparative separation of enantiomers for biological testing. Racemic Ro 31-9193 was successfully separated on a semi-preparative OD-R column (25 cm × 1 cm I.D.) using acetonitrile–0.1% aqueous trifluoroacetic acid (25:75) yielding mg quantities of the respective enantiomers. The IC_{50} (the amount of drug required to reduce activity of the enzyme to 50% of the uninhibited control) for the first-eluting enantiomer was 30 n*M* and 6 n*M* for the second-eluting enantiomer. The *trans* fused form of Ro 31-9193 was subsequently pursued as this was found to be more active than the *cis* configuration. Reversed-phase chiral chromatography would be compatible with drug metabolism/pharmacokinetics studies where monitoring of metabolites in aqueous media could be undertaken.

The OD-R column used for this work has been in use for over a year and subjected to various temperature changes and variations in mobile phase composition. Many samples have been assayed with little loss in performance. The separation of the racemate Ro 31-8830 has been used as a test for assessing the efficiency and chiral recognition of the Chiralcel OD-R column. The column is routinely washed with acetonitrile after use and capped off to prevent it drying out.

References

[1] Y. Nishizuka, *Nature*, 308 (1984) 693–698.
[2] A. Farago and Y. Nishizuka, *FEBS Lett.*, 268 (1990) 350–354.
[3] P. Davis, L. Elliott, W. Harris, C. Hill, S. Hurst, E. Keech, H. Kumar, G. Lawton, J. Nixon and S. Wilkinson, *J. Med. Chem.*, 35 (1992) 994–1001.
[4] K. Ikeda, T. Hamasaki, H. Kohno, T. Ogawa, T. Matsumoto and J. Sakai, *Chem. Lett.*, (1989) 1089–1090.
[5] A. Ishikawa and T. Shibata, *J. Liq. Chromatogr.*, 16 (1993) 859–878.