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# Direct chiral separation of almokalant on Chiralcel OD and Chiralpak AD for liquid chromatographic assay of biological samples

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## ABSTRACT

The four isomers of almokalant, a new antiarrhythmic substance under investigation, were separated by liquid chromatography on a Chiralcel OD and a Chiralpak AD column containing cellulose and amylose tris(3,5-dimethylphenylcarbamate), respectively. Both chiral stationary phases separate almokalant into the four isomers, but the retention orders are different if the carbamate is derivatized on cellulose or amylose. The Chiralcel OD column was used for the separation and determination of the isomers in urine at levels down to 100 nmol/l for the first three eluted and 200 nmol/l for the last with a relative standard deviation of less than 15%. The fluorescence response was increased by post-column ionization after stereoselective separation on the Chiralpak AD column. The isomers of almokalant could be determined at levels down to 10 nmol/l in plasma with a relative standard deviation of less than 15%.

## INTRODUCTION

Almokalant, 4-(3-{ethyl[3-(propylsulphinyl)propyl]amino}-2-hydroxypropoxy)benzonitrile (Fig. 1), is an antiarrhythmic drug substance under investigation in the early clinical phase. The compound contains one chiral carbon atom and one sulphoxide group in the molecule and is given as a stereoisomeric mixture of the four isomers. For pharmacokinetic purposes it is of interest to develop a liquid chromatographic method for the chiral separation of the four stereoisomers in biological samples. Almokalant contains an amino alcohol function and compounds of this kind have previously been separated on protein-based chiral columns, *e.g.*, Chiral-AGP, with good selectivity in many instances [1,2]. Recently the chiral stationary phases Chiralcel OD and Chiralpak AD, containing cellulose tris(3,5-dimethylphenylcarbamate) and amylose tris(3,5-dimethylphenylcarbamate), respective-

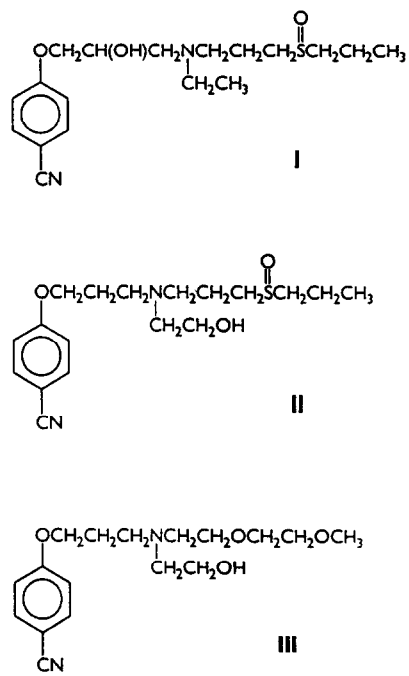


Fig. 1. Structures of almokalant (I) and the internal standards H 222/98 (II) and H 222/82 (III).

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ly, as a selector coated on to a macroporous silica gel have been used for the direct chiral resolution and assay of amino alcohol derivatives with β-adrenergic blocking activity [3,4]. The chromatographic conditions, such as type of alcohol and water content in the hexane mobile phase, influence the stereoselective properties of Chiralcel OD for amino alcohols [5,6]. Substances with a chiral sulphoxide group as in almokalant have been separated on Chiralcel (OB, OC, and OD) columns, as reported in the Application Guide from Daicel [7] and by Gaffney *et al.* [8]. The interactions of the polar carbamate group on the solute are different if the carbamate is derivatized on cellulose or amylose [9].

This paper reports the effects of the composition of the mobile organic phase on the stereoselectivity of Chiralcel OD and Chiralpak AD for the four isomers of almokalant. For assay in biological samples such as plasma a simple post-column reaction method was introduced to increase the detectability of the substance at least fivefold.

## EXPERIMENTAL

### *Chemicals*

Almokalant as racemate, the pure stereoisomers (*R*)-(−)H 262/15, (*R*)-(+)H 262/18, (*S*)-(−)H 262/20, and (*S*)-(+)H 262/21 and H 222/82 and H 222/98 (internal standards) in citrate buffer solutions (pH 5) were obtained from Medicinal Chemistry, Astra Hässle. The absolute configuration of the four isomers of almokalant is not yet known. The *R* and *S* refer to the carbon with the alcohol substituent (Fig. 1) and (+) and (−) to the optical activity of the sulphoxide group.

Hexane, 1-propanol, 2-propanol, acetonitrile and dichloromethane (HPLC grade) were from Rathburn (Walkerburn, UK) and diethylamine and trifluoroacetic acid from Fluka (Buchs, Switzerland). A Milli-Q system (Millipore, Molsheim, France) was used to supply deionized water. All buffer substances were of analytical-reagent grade (Merck, Darmstadt, Germany).

### *Instrumentation*

The liquid chromatographic system consisted of two LKB (Bromma, Sweden) pumps, a Kontron 460 autosampler (Tegimenta, Rotkreuz, Switzerland) and a Shimadzu (Kyoto, Japan) RF-551 fluorescence detector operated at 248 nm (excitation) and 306 nm (emission), cell volume 12 μl. A thermostatted bath (Lauda RMS, Königshofen, Germany) controlled the water temperature in the column jacket. The chromatograms were processed by a Multichrom version 1-8.2 chromatography data system from Fisons (Manchester, UK) or an SP 4400 integrator (Spectra-Physics, San Jose, CA, USA). A Metrohm (Herisau, Switzerland) 684 KF coulometer was used to measure the water content of the mobile phase. The analytical columns (250 × 4.6 mm I.D.), Chiralcel OD and Chiralpak AD from Daicel Chemical Industries (Tokyo, Japan), were used with flow-rates of 0.5 ml/min at a temperature of 35°C, unless stated otherwise. The mobile phase contained hexane with addition of 1-propanol, 2-propanol, acetonitrile and water, the composition being given in each experiment. All the mobile phases contained 0.1% diethylamine. Before use the Chiralpak AD column had to be conditioned for more than 24 h by passing mobile phase through it in order to remove fluorescent impurities that caused a high detector background.

For the analyses of biological samples a CN guard column (15 × 3.2 mm I.D., 7 μm) from Brownlee Labs. (Santa Clara, CA, USA) was used. A corresponding guard column with silica was tested as a mixing chamber for the post-column reaction. A Shodex DP-1 pulse damper was used between the pump and the T-tube to generate a backpressure. The T-tube was made in-house.

### *Test solutions*

Standard solutions of the substances were prepared in citrate buffer solutions (pH 5, $I = 0.1$). Test solutions in the organic mobile phase were prepared from alkalinized standard solutions by extraction with hexane–dichloromethane (1:1). After phase separation (freezing of the aqueous phase) and evaporation of the organic phase under nitrogen, the amines were dissolved in the mobile phase.

### *Analytical procedure*

Biological samples, 1.0 ml of urine or plasma, were alkalinized with 100 μl of 1 *M* NaOH, 100 μl of internal standard solution H 222/82 or H 222/98 (Fig. 1) were added and the mixture was extracted with 5.0 ml of hexane–dichloromethane (1:1) by shaking for 20 min. After centrifugation and freez-

ing of the aqueous phase, the organic phase was transferred into a test-tube and evaporated under a stream of nitrogen. The residue was reconstituted in 250 μl of the organic mobile phase by vigorous mixing and 100 μl of the solution were injected on to the column.

*Recovery and precision*

The absolute recovery of almokalant was determined by adding known amounts to blank plasma and processing the samples according to the analytical procedure. The chromatographic peaks were then measured and compared with those from injection of known solutions in the mobile phase.

The repeatability at different concentration levels was determined by assaying replicate samples ($n$ = 8) with added almokalant.

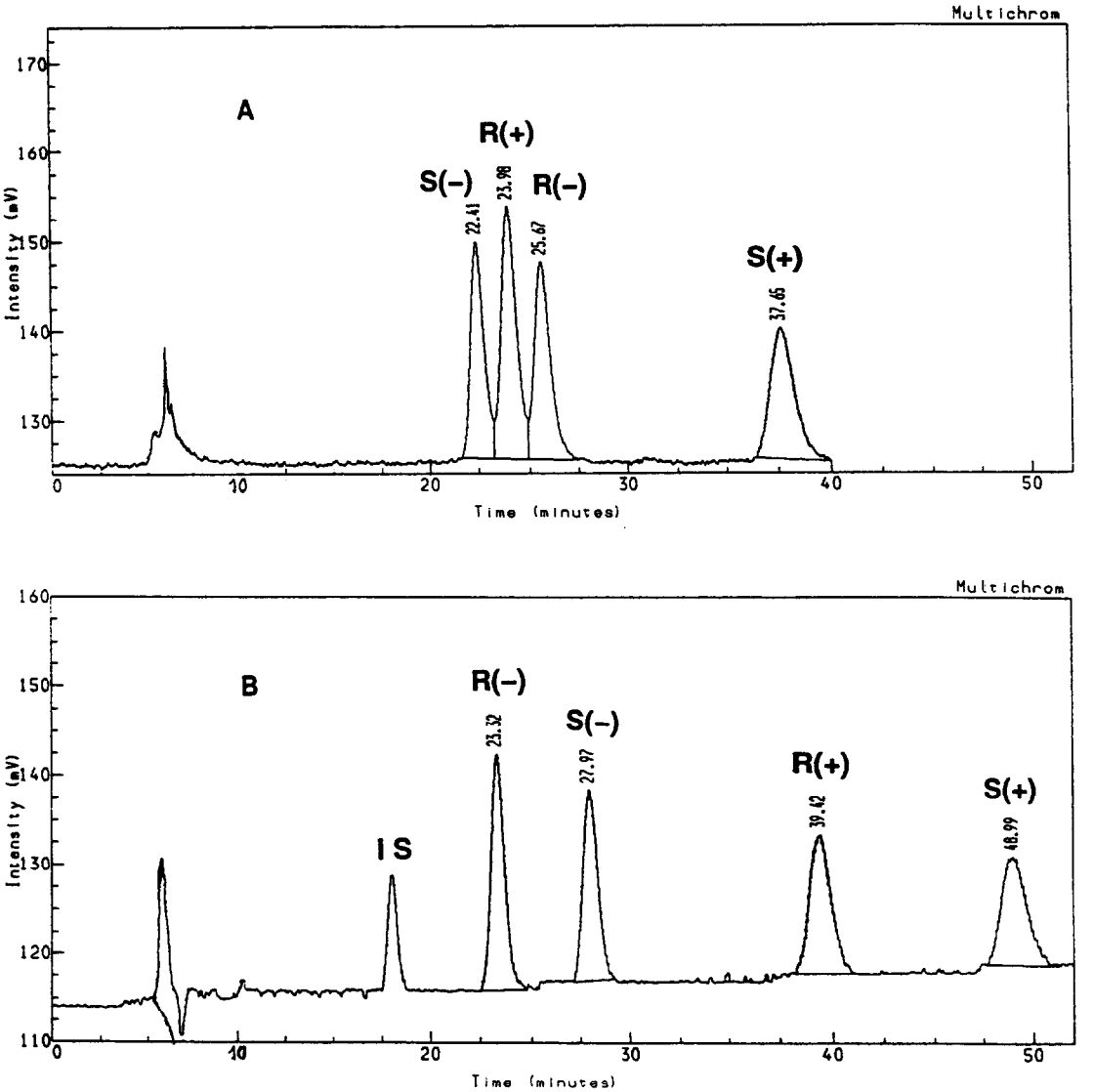


Fig. 2. Chromatograms for the separation of almokalant. Temperature, 30°C; flow-rate, 0.5 ml/min. (A) Chiralcel OD with a mobile phase of 15% 1-propanol–0.1% diethylamine–0.4 g/l water in hexane. (B) Chiralpak AD with a mobile phase of 14% 1-propanol–6% acetonitrile–0.1% diethylamine in hexane. IS = internal standard (H222/98).

## RESULTS AND DISCUSSION

### *Chiral separation*

We investigated different chromatographic systems for the resolution of the stereoisomers of almokalant. Previously Chiral-AGP with $\alpha_1$-acid glycoprotein as chiral selector was successfully used for the separation of the enantiomers of metoprolol [2]. Almokalant is also an aryloxyalkanolamine and is structurally related to a $\beta$-adrenoceptor blocking drug such as metoprolol, although it is more hydrophilic. On Chiral-AGP almokalant was incompletely resolved into two peaks at pH 6.5–7.5 (phosphate buffer) and it was not retained as much as desired. Other systems were also tested without success but on Chiralcel OD, cellulose tris(3,5-dimethylphenylcarbamate) on silica, also previously used for metoprolol [6], almokalant as base could be resolved into its four isomers. The elution order was (*S*)-(−), (*R*)-(+), (*R*)-(−) and (*S*)-(+) and the $\alpha$-values for adjacent peaks were 1.1, 1.1 and 2.6 (Fig. 2A). As for metoprolol and $\alpha$-hydroxymetoprolol [6], the water content of the mobile organic phase influences the stereoselectivity. The presence of a moderate amount of water in the mobile phase of 1-propanol and diethylamine in hexane decreased the retention of the (*R*)-(+) isomer, thus permitting separation. The addition of an excess of an aliphatic amine, diethylamine, is needed for peak symmetry, and an elevated temperature (35°C) and 1–2% of acetonitrile improve the column efficiency. This Chiralcel OD system was employed for stereoselective assay in urine (Fig. 3).

Chiralpak AD, amylose tris(3,5-dimethylphenylcarbamate), also separated the four stereoisomers but with a different retention order, (*R*)-(−), (*S*)-(−), (*R*)-(+) and (*S*)-(+), with $\alpha$-values for the adjacent peaks 1.3, 1.5 and 1.3 (Fig. 2B). The $\alpha$-value for the sulphoxide group, (*R*)-(+)/(*R*)-(−) and (*S*)-(+)/(*S*)-(−), is about 2.0 and for the chiral carbon, (*S*)-(+)/(*R*)-(+) and (*S*)-(−)/(*R*)-(−), about 1.3. The content of water in the organic mobile phase is not critical here but too large an amount of water will decrease $k'$ for the (*R*)-(+) isomer and also its separation from the (*S*)-(−) isomer. The Chiralpak AD column was used for the measurement of the almokalant isomers in blood plasma.

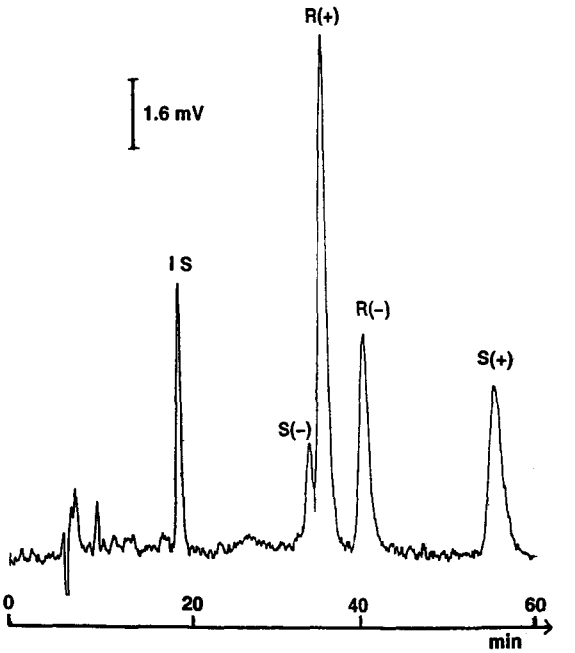


Fig. 3. Chromatogram from an authentic human urine sample on Chiralcel OD. Mobile phase, 7% 1-propanol–6% 2-propanol–1% acetonitrile–0.1% diethylamine–0.4 g/l water in hexane; temperature, 30°C; flow-rate, 0.5 ml/min. The concentrations of the isomers were 0.49 μmol/l (*S*)-(−), 1.26 μmol/l (*S*)-(+), 2.42 μmol/l (*R*)-(+) and 1.28 μmol/l (*R*)-(−). IS = internal standard (H 222/82).

### *Separation from urine samples on Chiralcel OD*

When applying the separation system, demonstrated in Fig. 2A, on an extract from a rat perfusate sample it was found that a metabolite of almokalant, the corresponding sulphone, interfered with the last-eluted (*S*)-(+) isomer on the Chiralcel OD column. By replacing some of the 1-propanol in the mobile phase with 2-propanol the (*S*)-(+) form was more retained and separated from this sulphone. A chromatogram of an authentic urine sample prepared according to the analytical procedure is shown in Fig. 3. The limit of quantitation for the three first eluted isomers was calculated to be 100 nmol/l and for the last isomer 200 nmol/l.

The selectivity of this method was checked by analysing a series of samples by an achiral reversed-phase method. The eluate fraction containing almokalant was collected and, after an additional extraction procedure, injected on to the Chiralcel OD col-

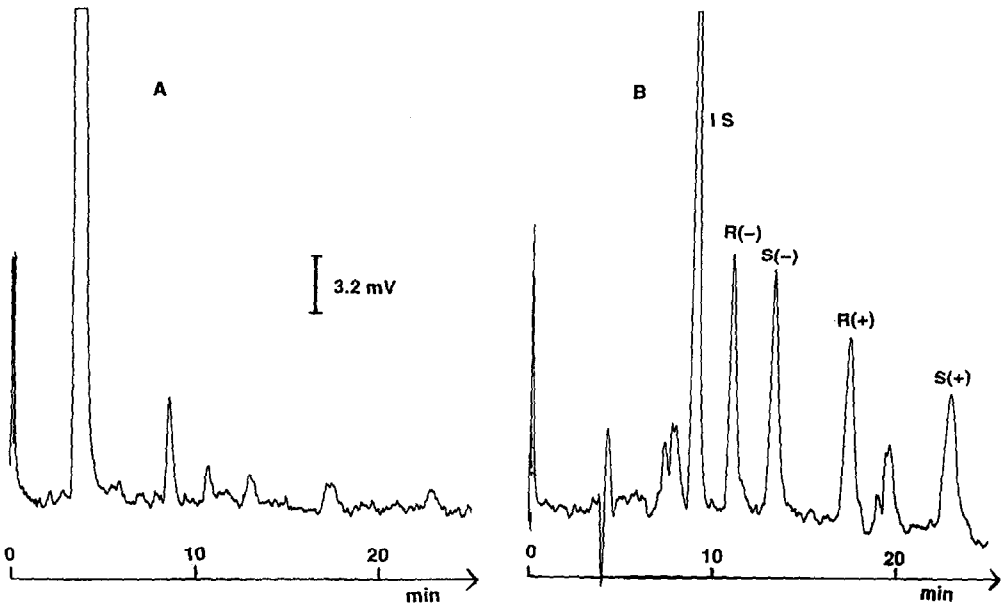


Fig. 4. Chromatograms from an authentic plasma sample containing 420 nmol/l almokalant on Chiralpak AD. Temperature, 35°C; flow-rate, 0.75 ml/min; mobile phase, 14% 1-propanol–6% acetonitrile–0.1% diethylamine in hexane, no extra water added. IS = internal standard (first-eluted isomers of H 222/98). (A) Mobile phase alone; (B) after postcolumn reaction with 0.3 mol/l TFA in hexane–ethanol (4:1) (0.15 ml/min).

umn. The results obtained confirmed those from the analytical procedure, without excessive sample work-up, as regards both the ratio between the different isomers and their individual concentrations. Studies on the stereoselective excretion of almokalant are in progress.

*Fluorescence amplification after separation on Chiralpak AD*

The fluorescence intensity of almokalant is much lower for the base form than for the protonated form, as was observed in the development of an achiral LC assay. The stereoselective separation of almokalant on Chiralcel OD or Chiralpak AD has to be performed under basic conditions, in the presence on an excess of diethylamine. However, the detector response was too low for the stereoselective assay of therapeutic concentrations in blood plasma samples. The more spacious separation pattern with the Chiralpak AD column enabled us to apply a post-column procedure to convert the resolved isomers into ionic form. The mobile phase flow of 0.75 ml/min from the analytical column was combined through a T-connection with a flow of 0.15 ml/min of trifluoroacetic acid (TFA) in hexane–ethanol (4:1). A Brownlee guard column with silica particles was inserted as a mixing chamber just before the inlet to the fluorscence detector, but it was later found to be unnecessary. By this procedure the fluorescence response of the four peaks of the almokalant isomers increased about eight-fold and the signal-to-noise ratio more than five-fold. There was a decrease in column efficiency of 10–20% but still satisfactory resolution. Chromatograms from an authentic plasma sample without and with TFA addition are shown in Fig. 4A and B, respectively. The separation on Chiralpak AD may also be used for the stereoselective assay of almokalant in urine samples. This has so far not been investigated as detection enchancement was not needed.

*Analytical validation*

In urine the isomers of almokalant could be measured at levels down to 100 nmol/l for the first three eluted peaks and 200 nmol/l for the last with a relative standard deviation of less than 15%. The calibration graph was linear between 100 nmol/l and 10

μmol/l. The repeatability (within-day) for the four isomers was about 1.6–3.5% at a level of 1.8 μmol/l and the reproducibility (between-day) was 3.5–5.0% at 2 μmol/l.

In plasma, concentrations down to 10 nmol/l could be measured with a relative standard deviation of less than 15%. The calibration graph was linear between 10 and 1000 nmol/l, which covers the expected therapeutic range. The repeatability for plasma samples was 5.4–17.8% at the 15 nmol/l level and 0.7–1.5% at 300 nmol/l of each stereoisomer. The reproducibility (between-day) was 2.1–4.3% at 100 nmol/l. The absolute recovery of almokalant from the biological samples was 100.5%.

The durability of the Chiralcel OD columns was found to be excellent and the same column could be used for more than 1 year, as reported earlier [6]. For Chiralpak AD one of the columns has been in use for more than 6 months with consistent performance. It has been possible to reproduce chiral separations from one column to the other provided that the temperature and water content of the mobile phase are controlled.

## NOTE ADDED IN PROOF

The absolute configuration of the four isomers of almokalant have recently been established: H 262/15 2*R*,3*R*; H 262/18 2*R*,3*S*; H 262/20 2*S*,3*R*; H 262/21 2*S*,3*S*.

## REFERENCES

1 G. Schill, I. W. Wainer and S. A. Barkan, *J. Liq. Chromatogr.*, 9 (1986) 641.
2 B.-A. Persson, K. Balmér, P.-O. Lagerström and G. Schill, *J. Chromatogr.*, 500 (1990) 629.
3 Y. Okamoto, M. Kawashima, R. Aburatani, K. Hatada, T. Nishiyama and M. Masuda, *Chem. Lett.*, (1986) 1237.
4 D. R. Rutledge and C. Garrick, *J. Chromatogr.*, 497 (1989) 181.
5 I. W. Wainer, R. M. Stiffin and T. Shibata, *J. Chromatogr.*, 411 (1987) 139.
6 K. Balmér, A. Persson, P.-O. Lagerström, B.-A. Persson and G. Schill, *J. Chromatogr.*, 553 (1991) 391.
7 *Applications Guide for Chiral Column*, Diacel, Tokyo, 1989.
8 M. H. Gaffney, R. M. Stiffin and I. W. Wainer, *Chromatographia*, 27 (1989) 15.
9 Y. Okamoto, R. Aburatani, Y. Kaida, K. Hatada, N. Inotsume and M. Nakano, *Chirality*, 1 (1989) 239.