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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON CHIRAL PACKED MICROBORE COLUMNS WITH THE 3,5-DINITROBENZOYL DERIVATIVE OF *TRANS*-1,2-DIAMINOCYCLOHEXANE AS SELECTOR*

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SUMMARY

Columns of small inner diameter have clear advantages over conventional columns, *e.g.*, reduced consumption of mobile phase and of expensive stationary phases, high column efficiency, enhanced mass sensitivity on concentration-sensitive detectors and easy interfacing with mass spectrometers. Columns with inner diameters of 4.0, 2.0 and 1.2 mm were prepared using a chiral stationary phase containing the 3,5-dinitrobenzoyl derivative of (*S,S*)(+)- or (*R,R*)(−)-1,2-diaminocyclohexane covalently bonded to the siliceous matrix. Two classes of racemic compounds of pharmaceutical interest were chosen to investigate the performance of chiral microcolumns: α-methylarylacetic acid anti-inflammatory agents (as 1-naphthalenemethylamides) and amino alcohol β-blocking agents (as oxazolidin-2-ones). These compounds were easily resolved (α = 1.21, 1.41 and 1.49 for ibuprofen, flurbiprofen and naproxen, respectively, and 1.40 for propanolol and pindolol) with very short analysis times. Hydrogen bonding, π–π interactions and steric hindrance are involved in the chromatographic resolution process.

INTRODUCTION

The technology of high-performance liquid chromatographic (HPLC) columns has recently been improved with respect both to the setting up of new chiral and achiral

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stationary phases and to the development of columns with different geometries. During our research on the direct resolution of enantiomeric mixtures by means of chiral stationary phases (CSPs), we obtained better results on packed microbore columns (I.D. 2.0 or 1.2 mm) than on traditional columns (I.D. 4.6–4.0 mm).

The development of micro-HPLC is mainly due to Scott and co-workers[1–4], Novotny and co-workers[5–9] and Ishii and co-workers[10–13]; most technological problems involved with this new technique pertaining to column packing, pumping and injecting systems and detection have been solved, at least for microbore columns (I.D. 0.5–2.0 mm), which can now be used in routine practice.

The main advantages achieved with the use of the above columns can be considered from two points of view, general and more specific. The general advantages are a considerable saving of mobile phase, an improved resolution of complex mixtures, easy interfacing with mass spectrometers, their compatibility with the small-scale manipulation required in modern biology and medicine and a higher sensitivity when the same amount of sample has to be injected when using concentration-sensitive detectors in sample-limited situations. Other advantages derive directly from the specificity of the selected columns, for instance, the possibility of using expensive and sophisticated mobile and/or stationary phases; in such instances, small volumes of mobile phase and small amounts of chiral stationary phases are necessary for good resolution.

In previous work on chiral stationary phases[14–16], we reported the design and preliminary evaluation of some that are particularly effective in the enantiomeric separation of many compounds of different classes. In this paper, a microbore column with a chiral stationary phase containing as selector the 3,5-dinitrobenzoyl derivative of (*S*,*S*)(+)- or (*R*,*R*)(−)-1,2-diaminocyclohexane (DACH-DNB), covalently bonded to the siliceous matrix, is described.

Two classes of products of interest from the pharmaceutical point of view were considered: racemic anti-inflammatory α-methylarylacetic acids and β-blocking amino alcohols. Recently the resolution of several derivatives of racemic α-methylarylacetic acids has been achieved using different systems and covalently bonded (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine[17], cellulose tribenzoate[18] and triphenylcarbamate[19] chiral stationary phases have been described.

Racemic β-blocking amino alcohols have generally been resolved as oxazolidin-2-one derivatives on several chiral stationary phases[20–22]. This paper reports the resolution of racemic ibuprofen, flurbiprofen and naproxen as 1-naphthalenemethylamides and of propranolol and pindolol as oxazolidin-2-ones on microbore chiral columns containing an (*S*,*S*)- or (*R*,*R*)-DACH-DNB as selector, and a critical comparison between the microbore and traditional columns.

The resolution of enantiomers of such compounds is of interest from the pharmaceutical point of view; it has been found that *R* isomers of ibuprofen[23] and naproxen[24] are converted *in vivo* into the corresponding *S* isomers; moreover, differences[25] in the disposition of the active *S* enantiomers of propranolol and its *R* enantiomer have been reported. Therefore, it is important to have a method of analysis that is rapid, sensitive and precise and that can be used for *in vitro* and *in vivo* studies of enantioselectivity in the biological activity of a racemic drug and to verify its enantiomeric composition; it is also important to be able to handle very small amounts of biological materials in sample-limited situations.

The most convenient approach to such a problem is direct resolution using chiral stationary phases (CSP); the indirect approach (derivatization with a chiral reagent) has some limitations owing to the possibility of partial racemization of the chiral derivative agent and of different reaction rates.

EXPERIMENTAL

Apparatus

HPLC was performed with a Carlo Erba Phoenix 20 syringe pump, a Knauer variable-wavelength UV detector (3-mm optical path length, 0.8-μl cell volume, time constant τ = 150 ms) and a Rheodyne Model 8125 5-μl loop injector. Chromatographic data were collected and processed on a Waters TM 840 data and chromatography control station.

Materials

LiChosorb Si 100 (5-μm particle size) and HPLC-grade solvents were purchased from Merck (Darmstadt, F.R.G.), 3-glycidoxypropyltrimethoxysylane (GOPTMS) from Janssen (Belgium); (*S,S*)(+)-DACH, (*R,R*)(−)-DACH, 1,1′-carbonyldiimidazole (CDI) and a 20% solution of phosgene in toluene from Fluka (Buchs, Switzerland), 1-naphthalenemethylamine from Aldrich-Chemie (Steinheim, F.R.G.). Racemic and enantiomerically pure drugs were obtained from the respective manufacturers and the remaining chemicals were of analytical-reagent grade and used as purchased.

The amides of α-methylarylacetic acids were synthesized by standard methods[17]. β-Blocking agents were converted into their oxazolidin-2-ones as described in the literature[21] or by reaction with CDI by the following precolumn derivatization micro-scale method. A 10-mg amount of propranolol hydrochloride (0.034 mmol) and 300 μl of triethylamine were dissolved in 2 ml of anhydrous tetrahydrofuran (solution A), and 10 mg of CDI (0.062 mmol) were dissolved in 2 ml of anhydrous tetrahydrofuran (solution B). Then 100 μl of solution A and 100 μl of solution B were mixed in a microvial and heated at 40°C and, after 5 min, the solution was injected into the HPLC system.

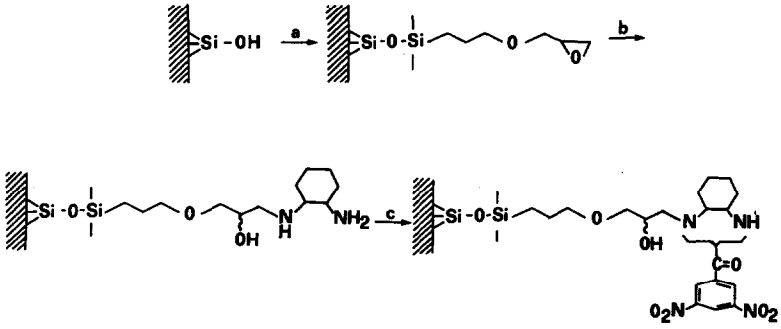

Fig. 1. Preparation of DACH-DNB chiral stationary phases. (a) Glycidoxypropyltrimethoxysilane, toluene, 4 h reflux; (b) (*S,S*)(+)- or (*R,R*)(−)-diaminocyclohexane, dimethylformamide, 96 h, room temperature; (c) dinitrobenzoyl chloride, tetrahydrofuran, triethylamine, 2 h reflux.

Chiral stationary phase

CSP was prepared as shown in Fig. 1.

Column packing

The column was made of stainless steel (150 mm × 4.0, 2.0 and 1.2 mm I.D.), packed with LiChrosorb Si 100 DACH-DNB (5 μm) using the slurry packing procedure.

Grafted silica (1.500, 0.700 or 0.200 g) was dispersed in chloroform (50, 10 or 5 ml, respectively) and then treated ultrasonically for 5 min. The slurry thus obtained was packed with a Haskel Model pump DSTV-122 using *n*-hexane as pressurizing agent.

Column evaluation

The eluent used in the evaluation of the kinetic performance of chiral columns of different inner diameter was *n*-hexane–isopropanol (99:1, v/v) and the solvent for the test mixture (benzene, naphthalene and anthracene) was *n*-hexane. The column dead volume (V_0) was determined from the retention of an unretained peak (benzene, using dichloromethane as eluent).

Dimensionless parameters such as reduced plate height (h), flow resistance parameter (ϕ), separation impedance (E) and reduced velocity (v) were calculated according to Bristow and Knox[26]; diffusion coefficients of solutes in the mobile phase were determined using the empirical Wilke–Chang equation[27].

RESULTS AND DISCUSSION

The direct resolution of racemates by HPLC needs the presence of an external chiral environment, which can be the stationary phase (CSP), the mobile phase or both. In these instances, the use of microbore columns permits a large reduction in the amount of chiral stationary and/or mobile phase, allowing the use of very sophisticated chiral sorbents and of "exotic" mobile phase; further, the amount of mobile phase necessary to optimize the separation conditions is dramatically reduced. Table I lists the amounts of CSP (DACH-DNB) used in the preparation of columns of different I.D.

The kinetic performance of the columns was evaluated especially on the basis of the results obtained using an achiral test (see Experimental); comparison of the Van Deemter curves obtained with the different columns is shown in Fig. 2. The flow-rates for optimum chromatographic efficiency of each column were determined and are

TABLE I

AMOUNTS OF CSP AND VOID VOLUMES FOR 150-mm LONG COLUMNS

I.D. (mm)	*Amount of chiral packing (mg)*	*Void volume (ml)*
4.0	900	1.60
2.0	225	0.40
1.2	80	0.14

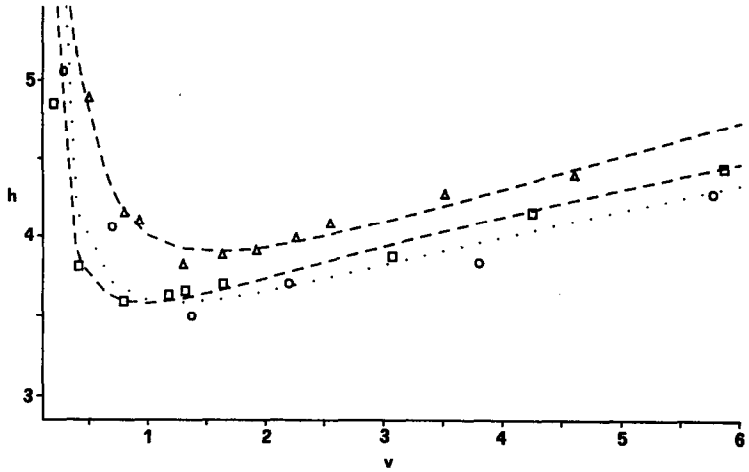

Fig. 2. Van Deemter plots (reduced plate height *vs.* reduced velocity of eluent) for different I.D. columns. (△) 4.0 mm I.D.; (○) 2.0 mm I.D.; (□) 1.2 mm I.D. See Table II for experimental conditions.

given in Table II. The results obtained show that, at mobile phase velocities close to optimum (v = 1.59, 1.37 and 0.80, respectively) the lowest h_{min} values, in the range 3.5–3.8, were obtained.

Although the lowest h_{min} value was obtained with the 2.0-mm I.D. column, the best performances (E value) were recorded with the 1.2-mm I.D. column. For this particular type of 5-μm irregular chiral stationary phase, the h_{min} value is higher than those previously reported in the literature for microcolumns containing 5-μm polar spherical stationary phases, but not very different from that reported for an NH_2 type of microcolumn which are the most difficult to optimize as far as the packing procedure is concerned[28].

The value of the reduced velocity (v) at which the best efficiency is registered decreases with decrease in the column I.D., in agreement with literature data[28].

An additional interesting result is the increase in the permeability of the columns as the I.D. decreases; it is therefore possible to connect several microbore columns in series, making feasible the resolution of mixtures of enantiomers with very low α values. Such resolution cannot be achieved with columns of standard dimensions.

TABLE II

COLUMN PERFORMANCES

Solute, benzene, k' = 0.15; eluent, *n*-hexane–2-propanol (99:1, v/v); CSP, (*S,S*)-DACH-DNB–LiChrosorb Si 100, 5 μm; column length, 150 mm.

I.D. (mm)	h_{min}	v_{opt}	Φ	E
4.0	3.8	1.59	760	10.900
2.0	3.5	1.37	622	7.620
1.2	3.6	0.80	538	6.970

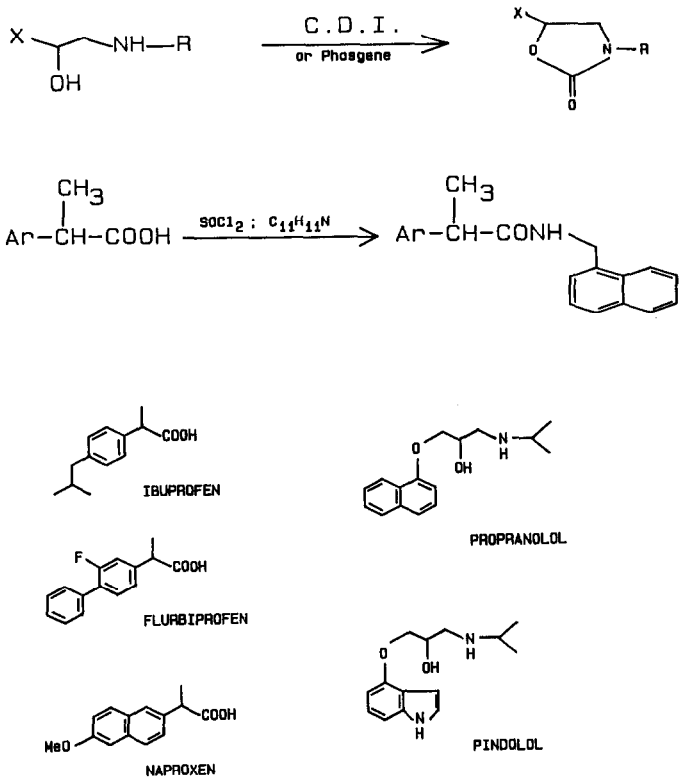

Fig. 3. Structures of racemic pharmaceuticals and derivatization procedures.

Chromatographic separation of chiral species

The above columns were successfully utilized in the direct resolution of racemates of substances of pharmaceutical interest, belonging to classes of racemic β-blocking amino alcohols and α-methylarylacetic acid anti-inflammatory agents. These substances were previously derivatized as shown in Fig. 3.

The derivatization reaction was carried out following previously described procedures[17,21] or, for β-blockers, using a micromethod of precolumn derivatization with CDI (see Experimental). The results reported in Table III show high values of α and relatively low capacity factors (k'), providing evidence for easy and rapid resolutions.

Fig. 4 compares the performances of analytical columns of different I.D. The signal-to-noise ratio (S/N) increases as the I.D. of the column decreases; in fact, when the same amount of substance is injected, S/N varies from 4.3 (B:C ratio) to 12.1 (A:C ratio) for the naproxen derivative, on passing from the 4.0-mm through the 2.0-mm to the 1.2-mm I.D. column, respectively.

The results indicate that microbore columns will be particularly useful in the biochemical field, when only small amounts of sample are available. Fig. 5 shows the analysis of a very small sample of the propranolol derivative at 230 nm using the 1.2-mm I.D. column.

TABLE III

CHROMATOGRAPHIC RESULTS

CSP: (*S,S*)-DACH DNB-LiChrosorb Si 100, 5 μm. All compounds were eluted at a linear velocity of 110 mm/s at 22°C. UV, detection 230 nm.

Compound	k'_1	α	R^*	*Eluent*
Ibuprofen	1.76 (*R*)	1.21	1.3	*n*-Hexane–2-propanol–dichloromethane (60:20:20, v/v)
Flurbiprofen	2.54 (*R*)	1.41	2.2	*n*-Hexane–2-propanol–dichloromethane (60:20:20, v/v)
Naproxen	4.11 (*R*)	1.49	3.4	*n*-Hexane–2-propanol–dichloromethane (50:25:25, v/v)
Propranolol	2.22 (*S*)	1.40	2.8	Dichloromethane–methanol (99:1, v/v)
Pindolol	1.36	1.40	2.7	Dichloromethane–methanol (90:10, v/v)

* Resolution factors obtained on a 150 × 1.2 mm I.D. column.

In addition, the utilization of microbore columns allows the determination of enantiomeric excess (e.e.) with an high degree of precision. In Fig. 6, as an example, the determination of the enantiomeric excess (99.94%) of (*S*)-propranolol, utilizing

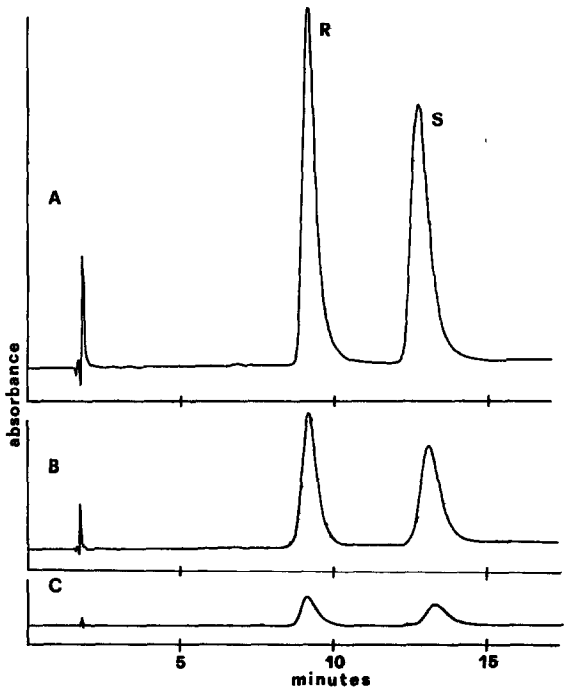

Fig. 4. Resolution of enantiomeric mixture of naproxen 1-naphthalenemethylamide. Injections of identical samples (0.2 μg) on to standard and microbore columns at similar linear velocities. See Table III for experimental conditions. (A) Column, 150 × 1.2 mm I.D.; flow-rate, 0.080 ml/min. (B) Column, 150 × 2.0 mm I.D.; flow-rate, 0.200 ml/min. (C) Column, 150 × 4.0 mm I.D.; flow-rate, 0.800 ml/min.

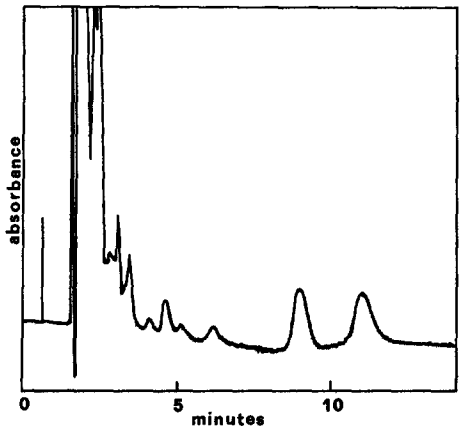

Fig. 5. Resolution of enantiomeric mixture of propranolol oxazolidin-2-one (1 ng). Column, 150 × 1.2 mm I.D.; eluent, *n*-hexane–2-propanol–methanol (40:30:30, v/v); flow-rate, 0.140 ml/min; UV detection, 230 nm; 0.01 a.u.f.s. $k'_1 = 7.30$; $\alpha = 1.30$.

a 1.2-mm I.D. column, is reported. Fig. 7a–c, showing some separation of enantiomers on 1.2-mm I.D. microbore columns, illustrates the potential of these columns.

In order to demonstrate the practical application of these columns to the control of the enantiomeric excess of chiral active principles in pharmaceutical specialities, a study of the precision and linearity of the detector response was carried out; the results are shown in Table IV and Fig. 8. The values of both the linear regression coefficients and the coefficients of variation indicate that the methodology is suitable for everyday routine use.

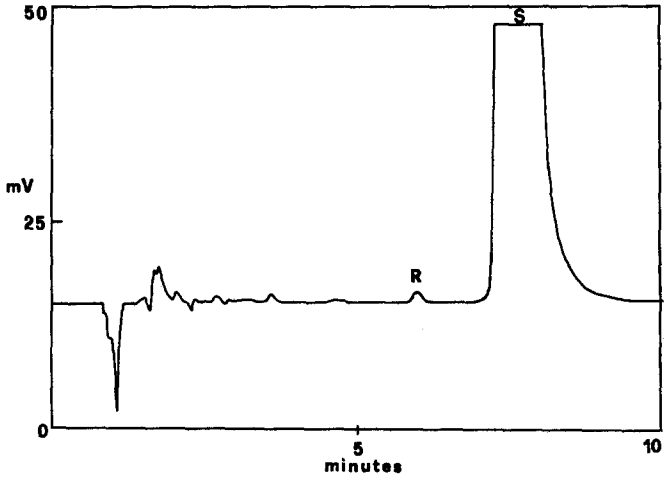

Fig. 6. Optical purity determination of (*S*)-propranolol (e.e. 99.94%). Column, 150 × 1.2 mm I.D., packed with (*R,R*)-DACH-DNB CSP–LiChrosorb Si 100, 5 μm; eluent, dichloromethane–methanol (99:1, v/v); flow-rate, 0.080 ml/min; UV detection, 230 nm; 0.04 a.u.f.s. $k' = 2.22$; $\alpha = 1.40$.

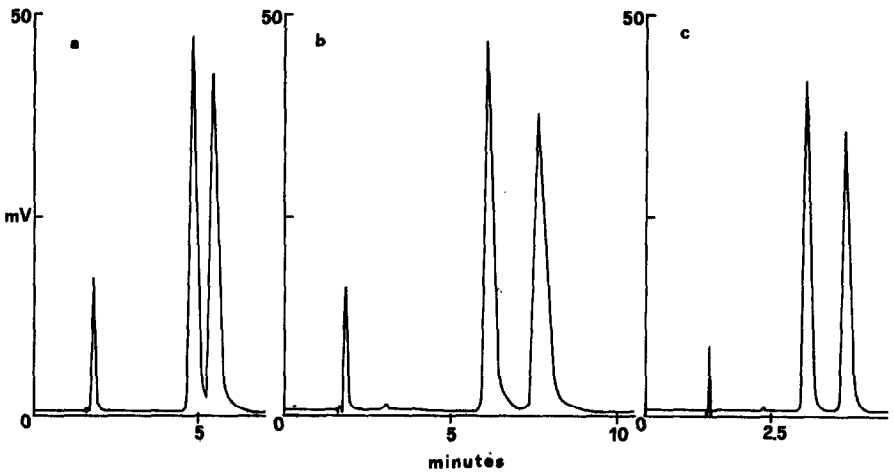

Fig. 7. Resolution of enantiomeric mixtures on a 150 × 1.2 mm I.D. column. See Table III for experimental conditions. (a) Ibuprofen 1-naphthalenemethylamide; (b) flurbiprofen 1-naphthalenmethylamide; (c) pindolol oxazolidin-2-one.

The mechanism of recognition assumes stereospecific attractive interactions between the racemic solutes and the selector through amide groups (hydrogen bonds) and 3,5-dinitrophenyl groups (π–π bonds) of the latter; further, the strong selector rigidity is partly responsible for the high values of α observed.

With derivatives of α-methylarylacetic acids, increases in the values of k' and α with increasing π-donor capacity of the aryl moiety have been observed, showing that it is directly involved in the mechanism of resolution through a π-base–π-acid interaction with the 3,5-dinitrophenyl groups present in the CSP.

TABLE IV

REPRODUCIBILITY STUDY

Sample: racemic propranolol oxazolidin-2-one. See Table III for experimental conditions.

Injection	*Area of 1st peak**	*Area of 2nd peak**
1	958 919	960 336
2	973 016	966 993
3	965 430	960 195
4	963 286	964 009
5	956 930	941 711
6	966 636	967 231
7	984 893	985 192
8	974 009	974 502
9	961 511	961 278
10	962 248	962 451

* 1st peak: mean value 966 688; standard deviation σ = 8001; coefficient of variation = 0.83%. 2nd peak: mean value 964 389; standard deviation σ = 10 544; coefficient of variation = 1.09%.

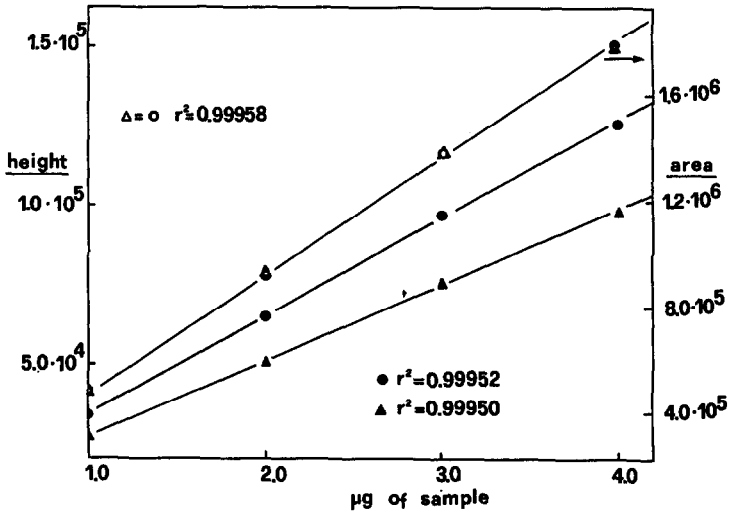

Fig. 8. Linear relationship between the amount of sample injected and the detector response. Solute: racemic propranolol oxazolidin-2-one. Column, 150 × 1.2 mm I.D. packed with (*R,R*)-DACH-DNB CSP–LiChrosorb Si 100, 5 μm; eluent, dichloromethane–methanol (99:1, v/v); flow-rate, 0.080 ml/min; UV detection, 230 nm; 0.16 a.u.f.s. (○) *R* isomer and (△) *S* isomer areas; (●) *R* isomer and (▲) *S* isomer heights.

The general mechanism of chromatographic resolution involves the temporary formation of CSP–solute complexes; the different stabilities of the complexes formed by the *R* and *S* isomers with the selector are determined by enthalpic and entropic factors. Under our experimental conditions, at room temperature, the chromatographic resolution is essentially an enthalpy-controlled process, whereas the entropic factor adversely affects the resolution. In fact, the more retained enantiomer is the one which simultaneously creates the greatest number of attractive interactions and therefore it is the one that loses the most degrees of freedom during the interaction with the CSP.

The reaction of propranolol and pindolol with phosgene or CDI produces cyclic derivatives with structural rigidity; this permits the disadvantageous effect of the entropic contribution on the enantioselectivity to be minimized and high values of α to be obtained, even in the presence of very polar mobile phases.

CONCLUSIONS

Recent progress in microcolumn instrumentation has made microbore packed chiral columns extremely useful for difficult separations of enantiomers. The possibility of working with very small amounts of substances permits studies of pharmacodynamics and pharmacokinetics on very small samples of precious biological fluids; further, the reduced amounts of chiral phases required in columns of small I.D. permit the utilization of extremely sophisticated and/or expensive chiral matrices.

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