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Direct high-performance liquid chromatographic separation of (+)- and (−)-medetomidine hydrochloride with an α_1-acid glycoprotein chiral column

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SUMMARY

The aim of this work was to find a fast and reliable separation method for the two enantiomers of the new animal drug medetomidine hydrochloride. Several attempts had earlier been made with commercially available chiral columns but with unsatisfactory results. A direct, fast and reproducible separation method using a commercially available α_1-acid glycoprotein column, Chiral-AGP, was developed for the complete separation of the two enantiomers. The chiral stationary phase was immobilized on porous spherical silica particles (5 μm), which enables high-performance liquid chromatography to be operated in the reversed-phase mode. The column showed good stability during several months of frequent use. The separation was performed with various mixtures of an organic modifier (1-propanol, 2-propanol, methanol, ethanol and acetonitrile) and phosphate buffer as the mobile phase. The effect of changing the mobile phase solvent ratio, temperature, flow-rate, buffer concentration and buffer pH on the capacity factors, selectivity and resolution was studied. The chromatographic conditions chosen for the separation permitted the rapid separation of the two enantiomers within 8 min.

INTRODUCTION

Medetomidine hydrochloride (Recommended International Nonproprietary Name, Rec. INN), (±)-4-[1-(2,3-dimethylphenyl)ethyl]-1*H*-imidazole hydrochloride (DOMITOR®; Farmos Group) (Fig. 1), a white, crystalline compound[1], is an α_2-agonist which has potent sedative and analgaesic effects[2–6] and is used to facilitate

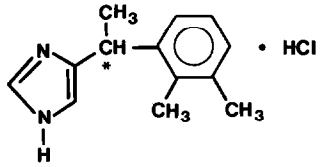

Fig. 1. Molecular structure of medetomidine hydrochloride.

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minor surgical and diagnostic procedures in dogs and cats. Intensive pharmacological studies with both enantiomers have been carried out in recent years and the results have shown that only the (+)-form, dexmedetomidine hydrochloride (Rec. INN), is pharmacologically active[7,8]. For future purposes, *e.g.*, to determine the purity of one enantiomer, it was necessary to develop a rapid and reliable direct method for the separation of these enantiomers.

It is possible to separate the two enantiomers by forming carbamate derivatives with ethyl chloroformate and to separate them by high-performance liquid chromatography (HPLC) using a Bakerbond Chiral Phase DNBPG covalent column (J. T. Baker, Phillipsburgh, NJ, U.S.A.)[9]. This very sensitive method has some drawbacks, however. The reaction time between medetomidine hydrochloride and ethyl chloroformate is fairly long (2–3 h) and the reaction also produces minor side-products, although these do not significantly interfere with the analysis.

A direct and a faster means of separating the two enantiomers of medetomidine hydrochloride was needed, and we decided to investigate the use of different chiral HPLC columns. First the "brush-type" column materials were studied[10–13]. In these commercial columns, N-(3,5-dinitrobenzoyl)leucine and D-phenylglycine are covalently bound to a spherical silica gel (5 μm) (Bakerbond Chiral Phase DNBLeu covalent, J. T. Baker, and Hi-Chrom reversible Pirkle leucine and L-phenylglycine covalent columns, Regis Chemical, Morton Grave, IL, U.S.A.). It turned out, however, that the interactions of the two enantiomers of medetomidine hydrochloride with the "brush-type" phases were similar and resolution was therefore not observed.

Experiments with commercially available cavity columns based on α-, β- and γ-cyclodextrin bound to a spherical silica gel (5 μm)[14,15] were performed (Cyclobond I, II and III, Advanced Separation Technologies, Whippany, NJ, U.S.A.). Resolution, based on the different strengths of the inclusion complexes of the enantiomers, was not achieved, however.

After these attempts, our interest turned to protein columns, which are known to be suitable for the separation of enantiomers of several drug substances. The separation mechanism of these protein columns is based on bioaffinity with different interactions. First a commercially available column based on bovine.serum albumin covalently bonded to a silica gel[16,17] (Resolvosil BSA-7, Macherey-Nagel, Düren, F.R.G.) was tried. Soon it turned out that a protein-type column could provide a solution to our problem. A promising qualitative separation between the two enantiomers of medetomidine hydrochloride was observed with this BSA column, but the separation was not sufficient for quantitative work (Fig. 2a).

Finally another commercially available protein column was chosen. This column was based on 10-μm diethylaminoethylsilica gel onto which α_1-acid glycoprotein has been immobilized by ionic binding and cross-linking[18–29] (EnantioPak cartridge, LKB, Stockholm, Sweden). With this column a good quantitative separation method for the two enantiomers was developed (Fig. 2b). Subsequently a second-generation α_1-AGP column (Chiral-AGP, ChromTech, Norsborg, Sweden) was tested. In this column the protein is immobilized on 5-μm silica particles by both cross-linking and covalent binding. A suitable separation method was developd with this column (Fig. 2c) and is discussed in this paper. A better resolution and improved column stability were observed compared with the columns mentioned above.

EXPERIMENTAL

Chemicals and materials

Analytical-reagent grade chemicals were used unless indicated otherwise.

2-Propanol was obtained from J. T. Baker (Deventer, The Netherlands), 1-propanol, KH_2HPO_4 and $Na_2HPO_4 \cdot 2H_2O$ from Merck (Darmstadt F.R.G.), acetonitrile and methanol of HPLC grade from Mallinckrodt (Paris, KY, U.S.A.) and ethanol from Alko (Rajamäki, Finland).

The water used was deionized, ultrafiltered, reverse osmosis water, prepared in-house.

The phosphate buffers used were prepared according to Sörensen.

The HVLP 0.45-μm filters used for mobile phase filtration were obtained from Millipore (Molsheim, France).

Apparatus

Chromatographic data were obtained using a Hewlett-Packard 1090 M liquid chromatographic system equipped with a built-in diode-array detector (HP 79880A), automatic sampling system (HP 79847A), variable-volume injector (HP 79846A), binary solvent-delivery system (HP 79835A) and analytical workstation (HP 79994A). The system was additionally equipped with a built-in oven and was connected to an HP ThinkJet printer and a HP ColorPro graphics plotter.

A Chiral-AGP (particle size 5 μm) column (10 cm $\times$ 4 mm I.D.) was used.

Chromatographic procedure

Racemic ($\pm$)-medetomidine hydrochloride and its pure enantiomers were dissolved in water at concentrations of 0.03 mg/ml. The volume injected was 20 μl and the detection wavelength was 220 nm. Different mobile phase compositions (mixtures of different organic modifiers and phosphate buffer) and parameters (mobile phase solvent ratio, temperature, flow-rate, buffer concentration and buffer pH) were changed in turn and the effects on the capacity factors (k'), selectivity (α) and resolution (R_s) were calculated from the recorded chromatograms using the equations

$$k' = t/t_a - 1$$
$$\alpha = (t_2 - t_a)/(t_1 - t_a)$$
$$R_s = 2(t_2 - t_1)/(W_1 + W_2) \approx (t_2 - t_1)/[W_{1(h/2)} + W_{2(h/2)}]$$

where t = retention time; t_a = retention time of non-retarded component (methanol); W = width of peak; $W_{h/2}$ = width of peak at half height; 1 and 2 = 1st and 2nd eluted component.

RESULTS AND DISCUSSION

The results are presented in Tables I–V. It can be seen that a rapid and complete separation of the two enantiomers can be achieved by using the Chiral-AGP column based on α_1-acid glycoprotein as a chiral stationary phase.

The results show that changing the temperature, flow-rate or buffer concentration does not have a very significant effect on the selectivity or resolution. Changing

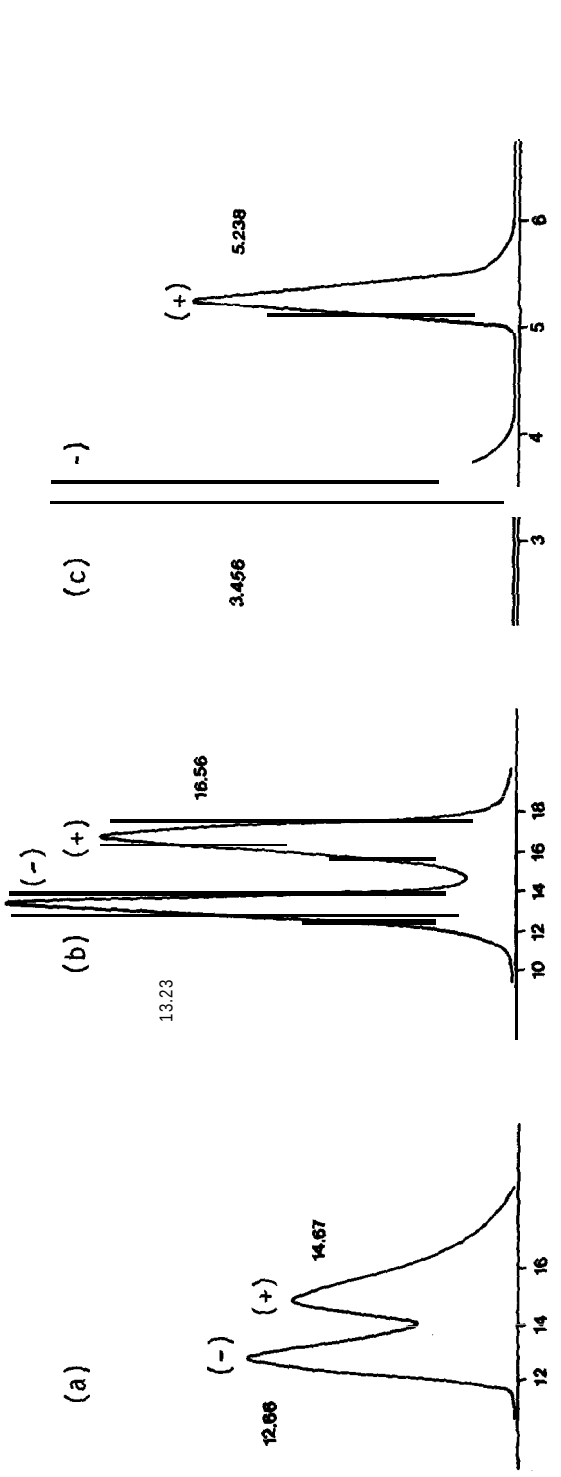

Fig. 2. Separation of the two enantiomers of medetomidine hydrochloride by using different protein-type chiral columns. (a) **Resolvosil** BSA-7. **Mobile phase,** 2-propano14.00.5 *M* phosphate buffer of **pH** 8.0 (2:98); flow-rate, 0.5 **ml/min;** temperature, ambient. **(b) EnantioPak** cartridge. Mobile phase, **2-propanol–0.005** *M* phosphate buffer of **pH** 6.5 (8:92); flow-rate, 0.3 **ml/min;** temperature, ambient. (c) **Chiral-AGP.** Mobile phase, aeetonitrileO.03 *M* phosphate buffer of **pH** 7.0 (17.5:82.5); flow-rate, 1.0 **ml/min;** temperature, ambient.

TABLE I

EFFECT OF MOBILE PHASE SOLVENT RATIO WHEN USING 2-PROPANOL AS ORGANIC MODIFIER ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, 2-propanol–0.03 *M* phosphate buffer of pH 7.0; flow-rate, 1.0 ml/min; temperature, ambient.

2-Propanol–buffer (v/v)	k'[a]	α	R_s
8:92	11.8	1.69	3.21
9:91	10.3	1.63	2.96
10:90	9.3	1.59	2.76
11:89	8.2	1.53	2.54

[a] Capacity factor of the second-eluted (+)-enantiomer.

TABLE II

EFFECT OF TEMPERATURE ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, 2-propanol–0.03 *M* phosphate buffer of pH 7.0 (10:90); flow-rate, 1.0 ml/min.

Temperature (°C)	k'[a]	α	R_s
Ambient (28)	9.1	1.52	2.54
30	7.7	1.48	2.43
35	6.4	1.43	2.26
40	5.4	1.39	2.08

[a] Capacity factor of the second-eluted (+)-enantiomer.

TABLE III

EFFECT OF FLOW-RATE ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, 2-propanol–0.03 *M* phosphate buffer of pH 7.0 (10:90); temperature, ambient.

Flow-rate (ml/min)	k'[a]	α	R_s
0.6	9.2	1.53	2.79
0.7	9.2	1.53	2.74
0.8	9.2	1.53	2.65
0.9	9.2	1.53	2.59
1.0	9.1	1.52	2.54

[a] Capacity factor of the second-eluted (+)-enantiomer.

TABLE IV

EFFECT OF BUFFER CONCENTRATION ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, 2-propanol–phosphate buffer of pH 6.0 (5:95); flow-rate, 1.0 ml/min; temperature, ambient.

Buffer concentration (M)	k'^a	α	R_s
0.005	13.3	1.17	1.29
0.01	10.7	1.22	1.40
0.02	8.2	1.25	1.43
0.03	7.9	1.29	1.59
0.05	6.8	1.31	1.67

[a] Capacity factor of the second-eluted (+)-enantiomer.

TABLE V

EFFECT OF BUFFER pH ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, 2-propanol–0.03 *M* phosphate buffer (8:92); flow-rate, 1.0 ml/min; temperature, ambient.

pH	k'^a	α	R_s
5.5	4.5	1.10	0.67
6.0	6.4	1.17	1.03
6.5	9.4	1.37	2.05
7.0	12.1	1.63	3.04
7.5	13.3	1.84	3.61

[a] Capacity factor of the second-eluted (+)-enantiomer.

the buffer pH, on the other hand, has a strong effect on all parameters. The higher the pH of the buffer, the better is the resolution of the two enantiomers. As the use of pH values higher than 7.5 for longer periods may decrease the column lifetime, owing to decomposition of the silica, a pH of 7.0 was chosen for routine analysis.

A phosphate buffer concentration of 0.03 *M* was selected as suitable buffer concentrations are 0.01–0.035 *M* and buffer concentrations above 0.03 *M* do not seem to have a significant positive effect on the capacity factor.

A flow-rate of 1.0 ml/min was chosen as higher flow-rates permit much faster separations, and this outweighs the fact that higher flow-rates give a slightly lower resolution.

Ambient temperature was used for analysis, as higher temperatures had a slight adverse effect on resolution.

When choosing the mobile phase solvent ratio for the analysis it was important to obtain a baseline separation of the two enantiomers without too long analysis times. In this instance 2-propanol–0.03 *M* phosphate buffer of pH 7.0 (11:89) gave a baseline resolution, but the ratio 10:90 was chosen for analysis to ensure a baseline separation even if the column performance declined.

The above chromatographic conditions selected for the separation of the two enantiomers of medetomidine hydrochloride permits a baseline resolution within 8 min.

TABLE VI

EFFECT OF MOBILE PHASE SOLVENT RATIO WHEN USING ACETONITRILE AS ORGANIC MODIFIER ON CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, acetonitrile–0.03 *M* phosphate buffer of pH 7.0; flow-rate, 1.0 ml/min; temperature, ambient.

Acetonitrile–buffer (v/v)	k' [a]	α	R_s
12.5:87.5	14.8	1.93	3.49
15.0:85.0	9.9	1.77	3.12
17.5:82.5	8.0	1.71	2.83
20.0:80.0	5.1	1.57	2.35
22.5:77.5	3.5	1.48	1.88

[a] Capacity factor of the second-eluted (+)-enantiomer.

So far the experimental part of this work had been carried out using 2-propanol as organic modifier. Other organic modifiers were also tested. 1-Propanol, methanol and ethanol separated the two enantiomers but not as well as 2-propanol. With acetonitrile, however, a better separation was achieved. The effects of changing the mobile phase solvent ratio when using acetonitrile as organic modifier on the capacity facors, selectivity and resolution are shown in Table VI. The results show that a better and faster resolution is achieved when using acetonitrile instead of 2-propanol as organic modifier. Acetonitrile–0.03 *M* phosphate buffer (17.5:82.5) permits a baseline separation.

The conditions chosen for analysis not only permit a baseline separation of the (−)- and (+)-enantiomers (with retention times of *ca.* 3.5 and 5.2 min, respectively),

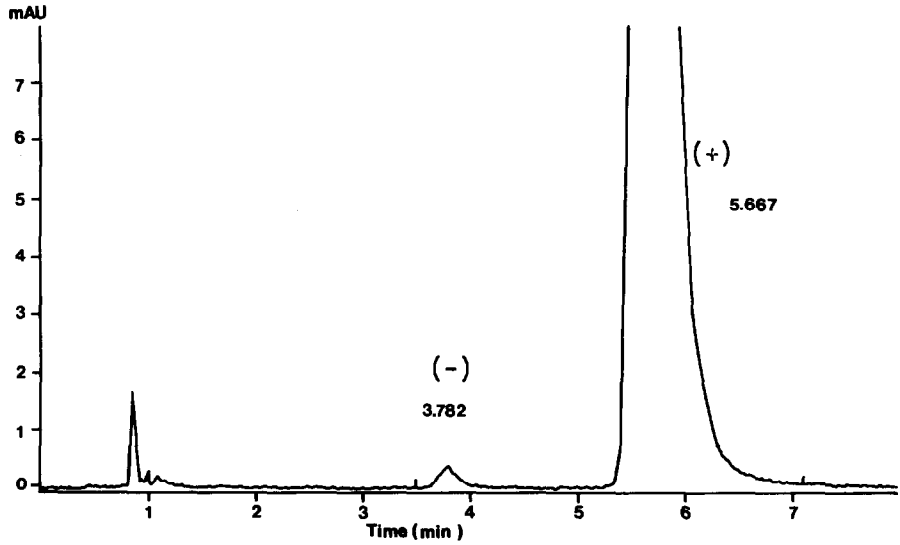

Fig. 3. Chromatogram of 0.5% (−)-medetomidine hydrochloride in (+)-medetomidine hydrochloride. Mobile phase, acetonitrile–0.03 *M* phosphate buffer of pH 7.0 (17.5:82.5); flow-rate, 1.0 ml/min; and temperature, ambient.

TABLE VII

STABILITY OF THE COLUMN BY COMPARING CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s)

Mobile phase, 2-propanol–0.005 *M* phosphate buffer of pH 6.0 (5:95); flow-rate, 1.0 ml/min; temperature, ambient. The filling of the column was done after the second check-point.

Time (months)	k' [a]	α	R_s
0.5	10.5	1.42	3.22
1.5	7.7	1.34	1.78
4.0	8.6	1.31	1.42
4.0[b]	10.3	1.39	1.72

[a] Capacity factor of the second-eluted (+)-enantiomer.

[b] Selectivity and resolution when the capacity factor is adjusted to the initial value (approximately) by changing the mobile phase solvent ratio to 4:96.

but as the peak shape is sharp and symmetrical it is also possible to determine very small amounts of (−)-medetomidine hydrochloride when present as an impurity in (+)-medetomidine hydrochloride, as shown in Fig. 3.

The Chiral-AGP column also showed satisfactory stability. During analysis the performance of the column and the symmetry of the peaks declined. The "shoulder" problem was solved by filling the empty space formed at the inlet end of the column with new sorbent, which restored the peak symmetry. The frequent and diverse use of the column inevitably had a slight negative effect on the column performance. However, by slightly changing the chromatographic parameters, *e.g.*, mobile phase solvent ratio or buffer pH, it is easy to maintain a rapid and complete separation of the two enantiomers even with a slightly deteriorated column.

The stability of the column (selectivity and resolution) can be seen in Table VII. The selectivity and resolution decrease the longer the column has been in use, which is due to a slight deterioration of the column. The capacity factor was higher at the 4-month check-point compared with the previous check-point, which is probably due to the filling of the column.

When the capacity factor of the second-eluted (+)-enantiomer after a 4-month interval is adjusted to approximately the same value as that at the initial check-point by changing the mobile phase composition, it can be seen how much worse the resolving ability of the column has actually become. It should be noted, however, that although the selectivity and resolution have decreased after 4 months of use, the performance of the column is still sufficient to perform a rapid baseline separation of the two enantiomers of medetomidine hydrochloride.

CONCLUSION

The separation of the two enantiomers of medetomidine hydrochloride turned out to be a difficult chromatographic problem. Only protein-type chiral columns showed a sufficient separation capacity. The Chiral-AGP column was found to be very suitable for the separation of the two enantiomers. With this column and the optimized

conditions [mobile phase acetonitrile–0.03 *M* phosphate buffer of pH 7.0 (17.5:82.5), flow-rate 1.0 ml/min and ambient temperature], it is possible to achieve a complete baseline separation of (+)- and (−)-medetomidine hydrochloride within 8 min. The narrow and symmetrical form of the peaks also makes it possible to determine small amounts of one enantiomer as an impurity in the other.

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