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HPLC enantiomer separation of a chiral 1,4-dihydropyridine monocarboxylic acid

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Abstract

An enantioselective anion exchanger based on *tert*-butylcarbamoylquinine as chiral selector and thiol-modified silica as chromatographic support was applied for the enantiomer separation of the shortacting calcium antagonist clevidipine after its hydrolysis to methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate. This hydrolyzed derivative of clevidipine is the primary metabolite and precursor of the parent drug. Method development included the steps of optimization of the composition of the mobile phase (pH, type and content of organic modifier, polar organic mode), screening of various structural analogs of above mentioned CSP, and evaluation of the effect of the flow rate. These detailed studies gave insight into the operational mode of the CSP, which is basically an enantioselective anion exchange mechanism. The polar organic mode turned out to be advantageous with regard to enantioselectivity and resolution. The optimized method makes use of an eluent composed of 0.125% acetic acid in acetonitrile with flow rate of 1 ml/min at a constant temperature of 25 °C, and allows the separation of the both enantiomers with enantioselectivity α of 1.25 and a resolution R_S of 3.0 within 10 min. On the above mentioned quinine carbamate phase the (*R*)-enantiomer is stronger retained, while a change to the corresponding quinidine carbamate CSP allows the reversal of the elution order.

Keywords: Methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate; Clevidipine; Felodipine; Calcium antagonist; Enantioselective high-performance liquid chromatography; Chiral anion exchanger; Cinchona alkaloid; Quinine; Quinidine; Chiral acid

1. Introduction

The target compound, methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (Fig. 1a) is the precursor and primary metabolite of the new ultrashort-acting dihydropyridine calcium antagonist clevidipine, butyroxymethyl methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (Fig. 1b). Moreover, it is also the precursor of the calcium antagonist felodipine, ethyl methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (Fig. 1c), which is in clinical use. Like the parent compounds, methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate possesses a stereogenic center in position 4 of the dihydropyridine ring. This has

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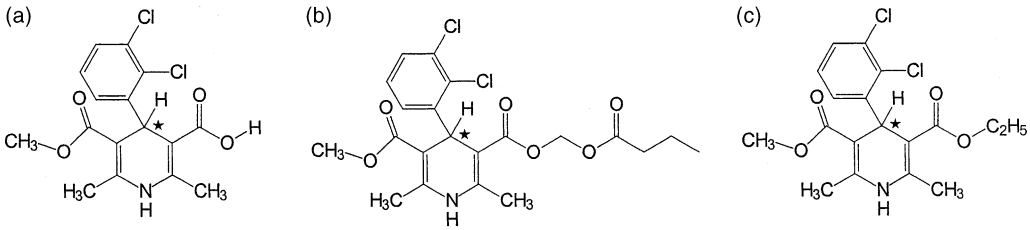

Fig. 1. Structure of (a) the solute methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate; (b) calcium channel antagonists clevidipine; and (c) felodipine that are derived from it.

impact inter alia on pharmacokinetics of clevidipine and requires stereoselective studies of its pharmacokinetic pathways as was discussed for other structurally related dihydropyridine type calcium antagonists [1]. For example, it was found a 10% difference between the in vitro hydrolysis rates of the enantiomers in human blood, which was attributed to a stereoselective protein binding [2].

There have been published a few reports on the enantiomer separation of the acid derivative of clevidipine. For example, chiral ion-pair chromatography with *Z*-L-arginine as counter-ion and Hypercarb as the stationary phase allowed baseline separation of the both enantiomers [3]. Chiralpak AD column was employed to separate the enantiomers of the target acid derivative by supercritical fluid chromatography with ultrashort analysis time, i.e. in less than 1 min [4]. The parent compound clevidipine on the other hand could be separated into individual enantiomers by enantioselective high-performance liquid chromatography (HPLC) with α_1-acid glycoprotein (AGP) column [5] and by SFC employing Chiralcel OD [4].

In this study, we present a new enantioselective method for the direct HPLC enantiomer separation of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate. The individual steps of method development are discussed in detail with the aim to figure out the major influential experimental parameters and display the major mechanistic peculiarities of the employed enantioselective anion exchange type CSPs [6–12] (Fig. 2). Finally, the elution order and the enantiomeric excess of an enantiomeric sample of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate was determined by application of the optimized method.

2. Experimental

2.1. Materials

Racemic methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate as well as racemate and (*S*)-enantiomer of clevidipine were obtained from the Department of Medicinal Chemistry of AstraZeneca, Mölndal, Sweden. The (*R*)-enantiomer of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate was obtained by hydrolysis of (*S*)-clevidipine with 0.1 M sodium

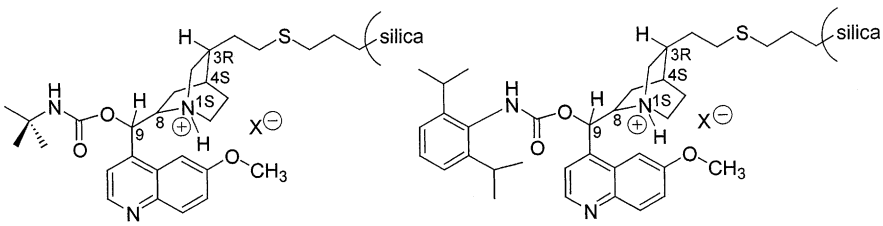

Fig. 2. Structures of the chiral stationary phases utilized for the present study.

hydroxide. Thus, 0.5 mg of (*S*)-clevidipine were dissolved in 0.3 ml of methanol, 0.5 ml 0.1 M sodium hydroxide solution were added and allowed to react at room temperature for 5 min. Then the reaction was stopped by adding 100 μl of 0.1 M HCl acid and 0.3 ml of the mobile phase to 100 μl aliquots of the reaction mixture. The final concentration of the sample was 0.125 mg/ml and aliquots of 100 μl were injected.

The chiral stationary phases, Prontosil Chiral AX QN-1, QD-1, QN-2, and QD-2 (Fig. 2) were from Bischoff Chromatography (Leonberg, Germany) and are also commercially available from Iris Technologies (Lawrence, KS, USA). Methanol and acetonitrile were of HPLC grade and supplied by VWR International (Vienna, Austria). Glacial acetic acid and ammonium acetate were of analytical grade and also from VWR International. The pH of the mobile phases was adjusted with glacial acetic acid and measured directly in the hydro-organic mixture thus representing the apparent pH (pH_a). The mobile phase was filtered through a 0.2 μm nylon membrane filter and degassed by sonication prior to use.

2.2. Instrumentation and HPLC method

HPLC experiments were performed with a Hitachi-Merck HPLC system which consisted of L-6200 intelligent pump, L-4250 UV-Vis detector, AS-2000A autosampler, D-6000 interface and HSM 7000 chromatography data station software from Merck (Darmstadt, Germany). The HPLC column was kept at a constant temperature of 25 °C with a column thermostat from W.O. Electronics (Langenzersdorf, Austria). Column dimensions were 150 mm × 4 mm i.d. The mobile phase compositions are specified in the tables and figures, respectively. Unless otherwise stated, the flow rate was 1 ml/min. The detection wavelength was set at 250 nm.

3. Results and discussion

3.1. Method development

3.1.1. pH-Optimization

The pK_a-values of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate are reported in the SciFinder Scholar database to be 1.71 (±0.20) and 7.01 (±0.20) (calculated by Advanced Chemistry Development, ACD, Software Solaris). Conversely, the pK_a of the quinuclidine nitrogen of the cinchona alkaloids is around 9 and 10 (depending on type of alkaloid and derivative). Thus at the typical working pH below 7, the quinuclidine is protonated. In the pH-range between 4.5 and 7 both selector and solute are ionized to a significant extent and interact with each other driven by ionic interaction so that a primary anion exchange retention mechanism is established under these conditions. Of course, the degree of dissociation of selector and analyte is then a major influential factor controlling retention and enantioselectivity. Therefore, a pH-profile was monitored using *tert*-butylcarbamoyl quinine based CSP and methanol–10 mM ammonium acetate buffer (80:20; v/v) as mobile phase. The resulting pH-curves are depicted in Fig. 3. Compared to other chiral carboxylic acid analytes lacking a weakly basic group as featured by the dihydropyridine nitrogen of the present solute the maxima and optima regarding retention factors and enantioselectivity, respectively, are shifted towards slightly higher pH. This was, according to the "net charge" concept, expected due to the amphoteric character of the analyte: a higher pH is needed for an amphoteric solute to achieve a similar negative net charge as for a comparable solute with acidic group only. It is seen that maximum retention is obtained at pH of 7, while at higher and lower pH the retention factors decrease. This behavior is typical for the ion-exchange process. In the pH-range above 7, the increasingly lower retention is a consequence of a reduced ionization of the weak anion exchanger which becomes the dominant factor for overall retention behavior. On contrary, below pH 7 the decline of retention factors is governed by the solute ionization which becomes less negatively charged as the pH is lowered (Fig. 3a). On the other hand, the optimal pH with regards to enantioselectivity is not obtained at maximum retention, but is slightly shifted to a lower pH-value so that the highest α-value is found at pH 6.0 (Fig. 3b). This is also the pH with the best resolution R_S between the individual enantiomer peaks.

3.1.2. Type and content of organic modifier

Upon the primary ion-exchange mechanism, a solvophobic retention mechanism is superimposed under reversed-phase conditions. Thus, the type and

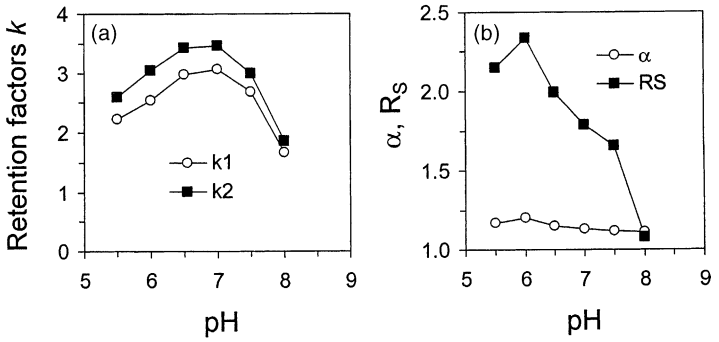

Fig. 3. pH-Profile of the separation of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate enantiomers. (a) Effect of pH on retention factors of first (○) and second eluted enantiomers (■); (b) effect of pH on enantioselectivity α (○) and resolution R_S (■). Conditions: CSP, *tert*-butylcarbamoyl quinine based CSP (Chiral AX QN-1); mobile phase, methanol–10 mM ammonium acetate (80:20; v/v); temperature, 25 °C; flow rate, 1 ml/min; other conditions see Section 2.

content of organic modifier will have a major influence on the chromatographic results. Both hydrophobic and dispersive interactions as well as electrostatics (through dielectric constant) are supposed to be affected by the content and type of organic modifier.

In accordance with the solvophobic theory the interaction of the solute enantiomers with hydrophobic areas of the selector and stationary phase, respectively, is increasingly weakened with addition of more and more organic modifier. This holds for both acetonitrile- and methanol-containing eluents that have a constant total buffer concentration (see Fig. 4a). It is however remarkable that at high modifier percentage (e.g. 70–90%) methanol possesses higher elution strength than acetonitrile, while with less modifier contents (e.g. 50%) acetonitrile has stronger eluotropic effect as was expected. This phenomenon may be attributed to the bimodal retention mechanism and the interrelated effect of the modifier on both, hydrophobic and dispersive interactions as well as electrostatic interactions including hydrogen bonding. Compared to acetonitrile, methanol is a significantly stronger competitor for intermolecular selector-solute hydrogen bonding due to its more pronounced H-donor and H-acceptor properties as well. This could explain the higher elution strength of methanol-rich eluents as compared to corresponding acetonitrile-rich eluents. More importantly, it is seen from Fig. 4b that the distinct solvatochromic solvent effects of the different modifiers, methanol and acetonitrile, are reflected in a strongly

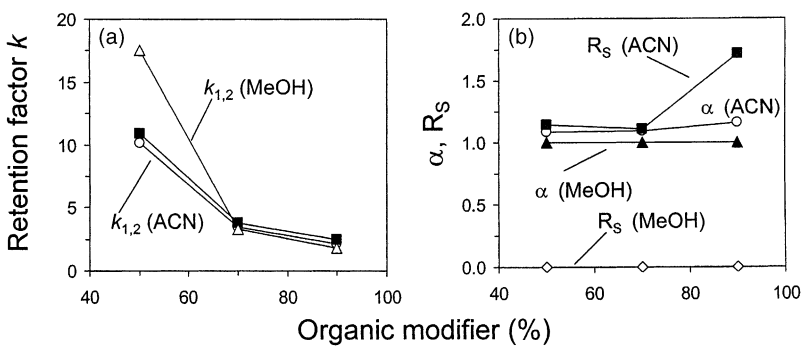

Fig. 4. Dependence of percentage of (a) organic modifiers methanol and (b) acetonitrile on retention factors and enantioselectivity as well as resolution. Conditions: CSP, *tert*-butylcarbamoyl quinine based CSP (Chiral AX QN-1); mobile phase, 10% 0.1 M ammonium acetate + 90% (water/organic modifier), pH_a 5.5; temperature, 25 °C; flow rate, 1 ml/min; other conditions see Section 2. Legend: (a) acetonitrile: k_1 (○), k_2 (■); methanol: $k_{1,2}$ (△); (b) acetonitrile: α (○), R_S (■); methanol α (▲), R_S (◇).

dissimilar effect on enantioselectivity. While a lack of enantioselectivity is noticed for methanol-containing eluents at any modifier content, acetonitrile-based mobile phases exhibit reasonable enantioselectivity at all percentages investigated. Enhanced α-values, though, are observed at higher acetonitrile contents, e.g. 1.16 at 80% versus 1.08 at 50% acetonitrile (see Fig. 4b).

The dependence of enantioselectivity and resolution on percentage of organic modifier points towards an even better separation under non-aqueous conditions. Therefore, also the polar organic mode was evaluated, as will be discussed in the next section.

3.1.3. Polar organic mode

In the polar organic mode non-aqueous conditions are adopted employing polar organic solvents such as methanol, acetonitrile, or mixtures thereof, as mobile phase with organic acids and/or bases as buffering constituents. Like with above discussed reversed-phase mode, the separation of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate on the quinine carbamate-based CSP in the polar organic mode follows basically also an enantioselective anion exchange mechanism. This also means that the retention may be readily balanced by the competitive effect of a counter-ion in the eluent. Here, acetic acid was used as counter-ion added to acetonitrile, methanol, and acetonitrile-methanol (50:50; v/v), respectively, in a concentration range of 0.125–0.5% (v/v) (22–87 mM) to balance the ionic interaction between the protonated selector and the analyte. Here it is pointed out that too low counter-ion concentrations must be avoided, as this would result in poor peak shapes leading eventually to peak splitting and similar phenomena. The results of the experiments are summarized in Table 1.

In the non-aqueous polar organic mode, solvophobic interactions are weak, while the electrostatic interactions are strengthened. Such conditions may be of advantage if the latter occur stereoselectively and the former non-stereoselectively. It is seen from Table 1 that indeed non-aqueous conditions are favorable over the hydro-organic mode, in particular those conditions with acetonitrile-based mobile phases. Acetonitrile has no H-donor activity and low H-acceptor properties. It therefore seems to interfere less with the selector-analyte complex formation than methanol or methanol-buffer mixtures that exhibit stronger H-bond donor and acceptor properties. The elution strength can be adjusted by the counter-ion concentration as mentioned above and seen from Table 1, whereby with decreasing acetic acid percentages in the mobile phase retention factors and enantioselectivity as well as resolution increase for the acetonitrile eluent. Similar behavior is observed in terms of retention for methanol and methanol–acetonitrile eluents. Overall, the optimized mobile phase was composed of 0.125% acetic acid in acetonitrile, which allowed the baseline separation of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate with a resolution (R_S) of 2.9. This mobile phase was employed for further studies.

3.1.4. CSP screening

An effective way to optimize enantioselectivity and to adjust the desired elution order is provided by a structural variation of the chiral selector. It has been shown that cinchona alkaloid-derived CSPs, which differ in the type of carbamate residue, show some complementarity with regard to enantioselectivity and thus applicability spectrum. In addition, corresponding quinine and quinidine carbamate CSPs usually

Table 1
Enantiomer separation results for methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate with polar organic modes[a]

Acetic acid		Acetonitrile (ACN)				MeOH				ACN–MeOH (50:50; v/v)			
% (v/v)	(mM)	k_1	k_2	α	R_S	k_1	k_2	α	R_S	k_1	k_2	α	R_S
0.5	87	2.04	2.36	1.16	1.77	0.64	0.72	1.13	0.64	0.38	0.6	1.58	0.71
0.25	44	3.16	3.81	1.21	2.42	0.73	0.9	1.23	1.03	n.d.	n.d.	n.d.	n.d.
0.125	22	4.48	5.36	1.25	2.93	0.87	1.03	1.18	1.09	0.68	0.92	1.35	0.89

[a] CSP, *tert*-Butylcarbamoylquinine-CSP; T, 25 °C; flow rate, 1 ml/min; detection, UV 250 nm.

Table 2
Enantiomer separation results for methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate on various cinchona alkaloid-derived CSPs[a]

CSP	k_1	k_2	α	R_S	N_1	N_2	e.o.[b]
tert-Butylcarbamoyl quinine	4.15	5.17	1.25	3.01	5000	4100	*S*
tert-Butylcarbamoyl quinidine	2.60	2.89	1.11	1.02	3400	2500	*R*
2,6-Diisopropylphenylcarbamoyl quinine	3.05	3.36	1.10	1.09	4000	3000	*S*
2,6-Diisopropylphenylcarbamoyl quinidine	3.21	3.33	1.04	<0.8	n.d.	n.d.	n.d.

[a] Conditions: Mobile phase, 0.125% acetic acid in acetonitrile; other conditions see Table 1.
[b] e.o., elution order; configuration of first eluted enantiomer.

exhibit reversed elution order, which is of practical relevance. Herein, the four CSPs illustrated in Fig. 2 were screened for their potential to separate the enantiomers of the target carboxylic acid compound using the same eluent. The results are summarized in Table 2. It is seen that the *tert*-butylcarbamoyl quinine-based CSP yields the highest α-value and resolution (R_S) among the investigated chiral anion exchanger variants ($\alpha = 1.25$; $R_S = 3.0$). Hence, the *tert*-butylcarbamoylquinine CSP was selected for further studies.

3.1.5. Flow rate

Enantiomer separation on present chiral anion exchangers involves multiple and simultaneously active strong specific non-covalent interactions which is associated with high affinity. A proper balance of these interactions is an ultimate requirement for a sufficient chromatographic performance. This can be easily achieved with the present CSPs, since they actually tolerate more or less all common chromatographic conditions including high organic modifier contents and ionic strengths and a wide pH-range (pH 2–8). On the other hand, a slow kinetics of interaction (in particular dissociation or desorption) is typical for such type of separations.

The Van Deemter plots, which are depicted in Fig. 5 may be interpreted in this sense. A large slope of the H/u-curves indicates a significant C-term contribution to band spreading, which may be explained inter alia by a slow desorption kinetics. As a consequence, a significant gain in efficiency may be the result when the linear flow velocity is lowered from standard flow rate of 1 ml/min to the minimum of the Van Deemter curves. Typical is an increase of the efficiency by a factor of 2. In the present case, an improvement factor of 2.3 is acquired for first eluted enantiomer and 2.1 for second eluted enantiomer for a change from 2 to 0.6 ml/min, which translates into a gain of resolution by a factor of about 1.5 (optimal $R_S = 3.42$) at the expense of speed of analysis, though. A minimum reduced plate height (h_{red}) of 4.66 is observed for the present separation, which is reasonable for an enantioselective column and separation system, respectively.

3.2. Application

(*S*)-Clevidipine was hydrolyzed with 0.1 M NaOH solution. Upon hydrolysis of the bis-ester derivative to the mono-ester the CIP-priorities of the substituents at the stereogenic center at C_4 of the dihydropyridine ring are changed (viz. the methyl ester has higher priority

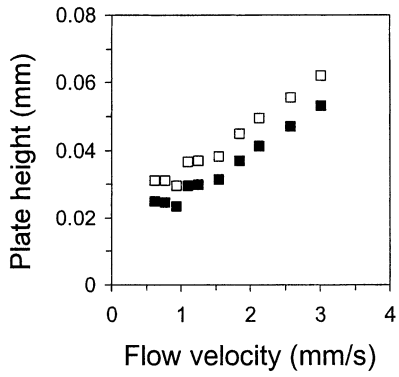

Fig. 5. Plots of height equivalents to a theoretical plate of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate vs. linear flow velocity (u) of the mobile phase. Conditions: CSP, *tert*-butylcarbamoyl quinine-modified Prontosil 120–5 μm (Chiral AX QN-1); mobile phase, 0.125% acetic acid in acetonitrile; other conditions see Section 2. First eluted enantiomer (■) and second eluted enantiomer (□). Volumetric flow rate varied between 0.4 and 2 ml/min.

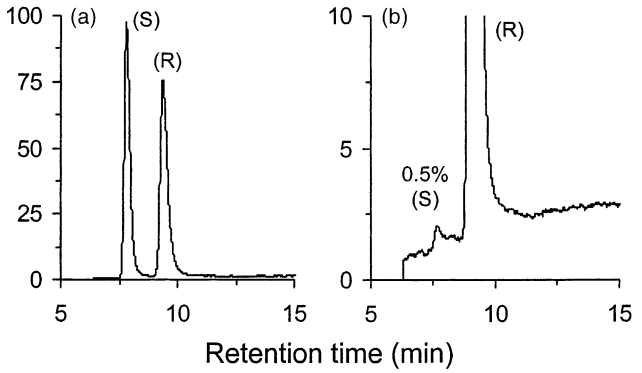

Fig. 6. Chromatograms of the racemate of (a) methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate and (b) its (*R*)-enantiomer on Prontosil Chiral AX QN-1. Conditions: Mobile phase, 0.125% acetic acid in acetonitrile; other conditions see Section 2.

than the free carboxylic acid group while it has lower priority than the butyryloxymethyl ester). Thus, the (*R*)-enantiomer of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate is obtained by hydrolysis of (*S*)-clevidipine.

It is seen from the chromatograms in Fig. 6a and b that the (*R*)-enantiomer of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate has higher affinity towards the *tert*-butylcarbamoyl quinine selector than the (*S*)-enantiomer which is eluted first. Hence the enantiomeric impurity of the (*R*)-enantiomer is eluted in front of the main peak, and can readily be detected and correctly quantified. In the given sample an enantiomeric impurity of 0.5% of (*S*)-enantiomer in (*R*)-methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate was determined which corresponds to an enantiomeric excess (ee) of 99%. To reach lower limits of quantitation of enantiomeric impurity a higher sample amount needs to be injected.

4. Conclusion

A new enantioselective method is presented that allows the baseline separation of methyl 4-(2′,3′-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate enantiomers, which is the primary metabolite of the shortacting calcium antagonist clevidipine. Several experimental parameters have been studied with the goal to end-up with optimized conditions and give some insights into the retention and separation mechanism of the enantioselective anion-exchange system. The optimized method makes use of the *tert*-butylcarbamoyl quinine CSP (Prontosil Chiral AX QN-1) and an eluent composed of 0.125% acetic acid in acetonitrile with flow rate of 1 ml/min at a constant temperature of 25 °C. It allows the separation of the both enantiomers with enantioselectivity α of 1.25 and a resolution R_S of 3.0 within 10 min. The method may be applied for stereoselective pharmacokinetic studies in biological samples such as plasma and urine, and/or enantiomeric purity control of clevidipine after its hydrolysis which takes less than 5 min until completion.

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References

[1] Y. Tokuma, H. Noguchi, J. Chromatogr. A 694 (1995) 181–193.
[2] H. Ericsson, B. Tholander, C.G. Regardh, Eur. J. Pharm. Sci. 8 (1999) 29–37.
[3] O. Gyllenhaal, A. Karlsson, Chromatographia 52 (2000) 351–355.
[4] O. Gyllenhaal, Fresenius J. Anal. Chem. 369 (2001) 54–56.
[5] A. Karlsson, A. Nyström, Chromatographia 53 (2001) 135–138.

[6] M. Lämmerhofer, W. Lindner, J. Chromatogr. A 741 (1996) 33–48.
[7] V. Piette, M. Lämmerhofer, K. Bischoff, W. Lindner, Chirality 9 (1997) 157–161.
[8] M. Lämmerhofer, N.M. Maier, W. Lindner, Am. Lab. 30 (1998) 71–78.
[9] A. Mandl, L. Nicoletti, M. Lämmerhofer, W. Lindner, J. Chromatogr. A 858 (1999) 1–11.
[10] N.M. Maier, L. Nicoletti, M. Lämmerhofer, W. Lindner, Chirality 11 (1999) 522–528.
[11] E. Zarbl, M. Lämmerhofer, F. Hammerschmidt, F. Wuggenig, M. Hanbauer, N.M. Maier, L. Sajovic, W. Lindner, Anal. Chim. Acta 404 (2000) 169–177.
[12] C. Czerwenka, M. Lämmerhofer, N.M. Maier, K. Rissanen, W. Lindner, Anal. Chem. 74 (2002) 5658–5666.