Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 875–880

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Short communication

In vitro study of enzymatic hydrolysis of diperodon enantiomers in blood serum by two-dimensional LC

K. Hroboňová [a], J. Lehotay [a,*], J. Čižmárik [b], D.W. Armstrong [c]

[a] *Department of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak Technical University, Radlinského 9, 812 37 Bratislava, Slovak Republic*

[b] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava, Slovak Republic*

[c] *Department of Chemistry, Gilman Hall, Iowa State University, Ames, IA 50011 3111, USA*

Received 19 January 2002; received in revised form 29 April 2002; accepted 18 May 2002

Abstract

An on-line coupled HPLC system is described for the determination of the enantiomers of diperodon in blood serum. The method involves three steps: (i) off-line preconcentration and clean-up, (ii) separation of the diperodon enantiomers from the matrix components on a reversed-phase stationary phase, and (iii) separation of the racemate from the reversed-phase column on a teicoplanin chiral stationary phase. The method is suitable for simultaneous determination of both enantiomers in serum up to 0.5 μg/ml. The degradation of diperodon enantiomers was studied in serum by an in vitro method and the experimental rate constants were determined. The enantiomeric hydrolysis rates and half-lives for diperodon in serum are different.

Keywords: Diperodon; Enantiomers; HPLC; Serum; Teicoplanin column; Enzymatic hydrolysis

1. Introduction

Diperodon (Fig. 1) (3-1-(piperidino)-bis-phenylcarbamate hydrochloride) is one of several phenylurethane derivatives of dialkyl amino alcohols which have demonstrated significant local anaesthetic activity [1,2]. The compound has a chiral centre and is administered clinically as the racemate.

Liquid chromatography has been shown to be well suited for the separation and determination of the enantiomers of diperodon. The optimisation of the separation of enantiomers of basic drugs (including diperodon) on an α_1-acid glycoprotein bonded stationary phase has been described by Schill et al. [3] and Hermansson and Grahn [4]. Other chiral stationary phases suitable for the separation of enantiomers of phenylcarbamic acid were based on the use of cyclodextrins [5] and cellulose derivatives especially cellulose tris-3,5-triphenylcarbamate [6]. Some π-association type columns were also reported to be suitable for achieving chiral separation of derivatives of phenylcarbamic acid [7,8]. Macrocyclic antibiotics form one of the most rapidly growing classes

* Corresponding author. Fax: +41-22-529-26043
E-mail address: lchotay@cvt.stuba.sk (J. Lehotay).

0731-7085/02/$ - see front matter
PII: S0731-7085(02)00347-3

of chiral selectors. They are known to resolve a variety of racemic compounds. Interactions that are hydrophobic, hydrogen bonding, dipole, π–π, steric repulsion and ionic in nature can occur between these chiral stationary phases and chiral analytes [9,10].

In this paper we describe an on-line coupled column liquid chromatographic system to determine the enantiomers of diperodon in serum. The method involves three steps: (i) an off-line clean-up and pre-concentration step, (ii) analytical separation of diperodon racemate from the matrix on a reversed-phase column, and (iii) the separation of the enantiomers from the analytical reversed-phase column on a teicoplanin chiral stationary phase. The developed method was used for study of in vitro enzymatic hydrolysis of diperodon enantiomers in rabbit blood serum. The results can apply to human serum because there is no significant difference in pharmacokinetic or pharmacodynamic properties [11].

2. Experimental

2.1. Materials

The diperodon was prepared at Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Comenius University in Bratislava. The organic solvents methanol, acetonitrile and triethylamine were of analytical grade (Merck, Germany), acetic acid was also analytical quality (Lachema, Czech Republic).

The packing of the off-line cartridge was C18, Sep-Pak Vac 3cc (200 mg) (Waters, Germany). For the achiral column Separon SGX C18 5 μm (30×3.2 mm I.D.) or (150×3.2 mm I.D.) (Tessek, Czech Republic) was used and the chiral column was a teicoplanin (Chirobiotic T, 250×4.6 mm I.D., 5 μm) (Astec, USA).

2.2. Off-line procedure

A 0.5 ml sample of blood serum was spiked with 50 μl of aqueous solution containing diperodon (concentration 0.1 mg/ml) and injected on Sep-Pak cartridge. Before use, the sorbent was conditioned with 2 ml of methanol, 2 ml of acetonitrile, and 10 ml of water. Then the sample was passed through the sorbent layer and washed with 1 ml of water and then 1 ml of acetonitrile. The diperodon retained by the sorbent was eluted with 1 ml of mixture methanol/acetonitrile/acetic acid/triethylamine 75/25/0.3/0.2 v/v/v/v. A 50 μl sample of eluate was injected into the reversed-phase column.

2.3. On-line procedure

The achiral column was conditioned with mobile phase (see Section 2.4) before injection of the serum extract or diperodon solution. After injection the achiral column was flushed with mobile phase for 3 min. Then the achiral column was connected to the chiral column and diperodon racemate was eluted in the back-flush mode with the mobile phase of the Chirobiotic T column (see part Section 2.4).

The concentration of the diperodon enantiomers was calculated from the total racemate concentration found on the achiral column and from the ratio of the peak areas of the enantiomers determined on the chiral column.

2.4. Chromatographic separation

The measurements were carried out with Hewlett–Packard HPLC system (series 1100) including a quaternary pump, a Rheodyne injection valve, and a photodiode array detector. With the off-line procedure the diperodon was extracted from serum and subsequently focused on the achiral C18 column, using a mixture of methanol/acetonitrile 45/55 v/v as mobile phase. For the chiral column, the mobile phase methanol/acetonitrile/acetic acid/triethylamine 45/55/0.3/0.2 v/v/v/v was used. The flow rates for the achiral and chiral columns were set at 0.5 ml/min, respectively. In-

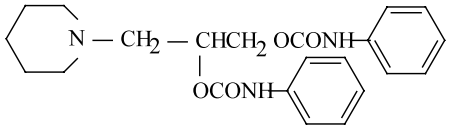

Fig. 1. Structure of diperodon.

jection volume was 50 μl and for detection, a photodiode array detector set at 240 nm was used.

2.5. Identification of enantiomers

For the measuring of optical rotation, the polarimeter Polar LμP (Na lamp, $\lambda = 589$ nm) (IBZ Messtechnik) was used. After separation, the fractions of enantiomers were collected to measure their optical properties. The preconcentrations of enantiomers in fractions were done by using the evaporation in nitrogen stream.

The elution order of enantiomers was determined by measuring the optical rotation of each peaks after HPLC separation of diperodon racemate. The first eluted enantiomer of the diperodon rotated the plane of polarised light to the right (+) and the second eluted enantiomer shows the opposite rotation.

3. Results and discussion

When designing an on-line coupled LC column system one is faced with problems concerning the compatibility of the mobile phases and dispersion of the sample. Usually the individual separation steps can be well optimised by tuning the mobile phase composition. However, the on-line transfer of the sample in one mobile phase to another mobile phase can destroy the separation on the second column. Therefore, in practice, usually a compromise in the mobile compositions must be made.

In order to optimise our on-line two column system, the effects of the mobile phase composition and the column dimension on the chromatographic resolution of enantiomers have been investigated.

3.1. The teicoplanin chiral stationary phase

As is evident from previous work the polar organic mode is suitable for the separation of enantiomeric derivatives of phenylcarbamic acid on macrocyclic glycopeptide chiral stationary phases [12]. Compounds with a minimum of two polar functional groups capable of relatively strong interactions with the chiral stationary phase can be enantioseparated in the polar organic mode. At least one of the polar functional groups must be on or near the stereogenic centre. The other polar group can be located anywhere in the molecule. The interactions are usually hydrogen bonds, but can sometimes be dipolar and/or electrostatic in nature [13,14]. The organic modifier concentration and amount of acid and base in mobile phase appear to be the most important parameters used to adjust the selectivity and retention on teicoplanin chiral stationary phases in the polar organic mode. In most cases methanol or acetonitrile is used as modifier.

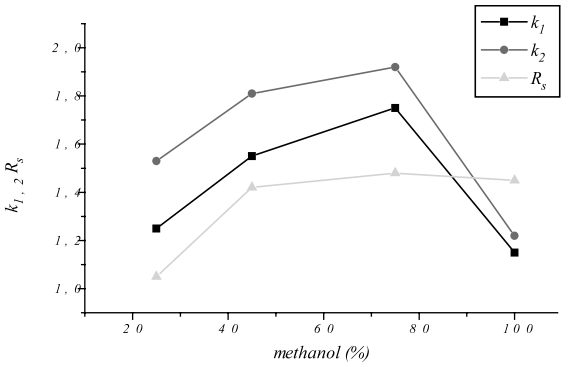

Fig. 2. Effect of methanol on k_1, k_2 and R_s of the diperodon enantiomers on teicoplanin chiral stationary phase. Conditions: mobile phase: methanol/acetonitrile/0.3% acetic acid/0.2% triethylamine; flow rate: 0.5 ml/min; injection volume: 50 μl; detection: UV, 240 nm.

For diperodon, the effect of the concentration of methanol on the enantiomeric retention factors (k_1 and k_2), and the values of resolution R_s were investigated and the results are given in Fig. 2. From this figure it can be seen that the methanol concentration has a pronounced effect on the values of the retention factors, but only a small influence on the resolution of these enantiomers. From these observation it can be concluded that good conditions for the separation of the enantiomers of diperodon are obtained using a mobile phase of methanol/acetonitrile/acetic acid/triethylamine 45/55/0.3/0.2. Fig. 3 shows a typical chromatogram of the diperodon enantiomers under these conditions.

3.2. The achiral column

The achiral reversed-phase column served two functions. It focused the diperodon at the top of the column and it separated the racemate from the rest of the matrix compounds. Subsequently, the concentrated enantiomers of diperodon were eluted on the chiral teicoplanin column. This approach could be used with repeated injections of the sample on the achiral column in case the concentration of diperodon was below the limit of detection. The limit of detection of diperodon with this system after preconcentration of 200 μl (4 × 50 μl) was approximately 30 ng/ml.

From a previous report on the analysis of diperodon by reversed chromatography, good retention ($k \sim 2.5$) and symmetrical peaks were obtained with a mobile phase mixture of methanol/water (80/20) to which 6.8 g/l of sodium acetate trihydrate was added [15]. However, this mobile phase was found to be unsuitable for the enantiomeric separation of diperodon on the teicoplanin column because injection of the diperodon solution (from the C18 column) destroyed the separation of the enantiomers on the chiral stationary phase. Therefore, a mobile phase was used in which the diperodon is not eluted from the achiral column (but is focused at the top of the column) while the matrix components are eluted quickly. The mobile phase for the achiral column was methanol/acetonitrile 45/55 (v/v) which is completely compatible with the mobile phase used for enantioseparation on the chiral teicoplanin chiral stationary phase. In order to desorb the diperodon enantiomer from the achiral C18 column, a small amount of acetic acid and triethylamine is added to the mobile phase (as described in the previous section and Section 2).

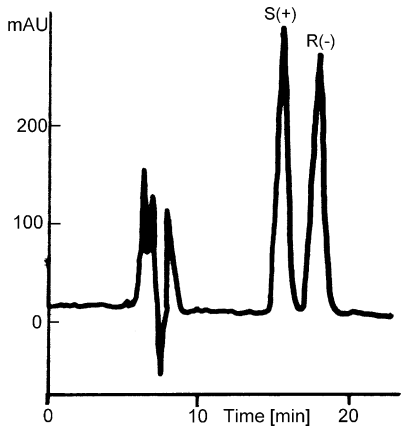

Fig. 3. Separation of diperodon enantiomers on teicoplanin chiral stationary phase. Conditions: mobile phase: methanol/acetonitrile/acetic acid/triethylamine 45/55/0.3/0.2 v/v/v/v; flow rate: 0.5 ml/min; sample: 5 μg/ml of diperodon; injection volume: 50 μl; detection: UV, 240 nm.

Table 1
The influence of the length of the achiral column on the total time of analysis, the values of the resolution of diperodon enantiomers (R_s), and number of theoretical plates (N) on teicoplanin chiral stationary phase

	Length of achiral column (mm)	
	150	30
Total time of analysis (min)	26–28	19–20
R_s[a]	0.9	1.5
N[a]	$(2.5–2.8) \times 10^3$	$(2.7–2.9) \times 10^3$

[a] The values of R_s and N was calculated for separation of enantiomers on the teicoplanin chiral stationary phase.

The effect of the achiral column dimension on the total time of analysis, the values of the resolution of diperodon enantiomers (R_s), and the efficiency of the separation (N) is shown in Table 1. By using the shorter achiral column, the total elution time was reduced and it had a positive effect on the number of theoretical plates for the enantiomeric separation and on the resolution values of the enantiomers. This was because band broadening in the shorter achiral column was less.

3.3. The performance of the on-line coupled two column system

The performance of the on-line coupled system was investigated with standard solutions of diperodon racemate and spiked rabbit blood serum samples. The calibration with standard solution and spiked serum in the range of 0.1–100 μg/ml (6 points) appeared to be linear for standard solutions ($r = 0.99$) and for spiked serum ($r = 0.98$). The enantiomer ratio with standard solu-

tions was found to be 1.02 ± 0.06 ($n = 15$) and for serum 1.13 ± 0.07 ($n = 10$). The limit of detection ($S/N = 5$) with standard solutions was approximately 0.1 μg/ml and for serum 0.5 μg/ml (50 μl was injected on achiral column). The recovery from the off-line clean-up of diperodon from spiked serum at a concentration level 10 μg/ml was found to be 85% with a R.S.D. of 3% ($n = 5$).

Fig. 4 shows the chromatograms of standard solutions of diperodon and spiked blood serum obtained on the coupled achiral and chiral stationary phases.

3.4. In vitro kinetic study

The degradation of diperodon is caused by various esterase enzymes. The developed HPLC method was applied to investigate the kinetics of enzymatic hydrolysis of the diperodon enantiomers. Diperodon in blood serum via the action of hydrolases, is decomposed to aniline, carbon dioxide, and piperidine-1,2-propandiol. The standard solution of diperodon racemate was added to the blood serum ($t = 0$) and the samples were incubated at 37 °C (time intervals from 0 to 6 h). The time course of the in vitro degradation of enantiomers of diperodon in blood serum is given in Fig. 5. This curve shows the difference in the concentration of the enantiomers after the incubation treatment, as well as the change in the enantiomeric ratio with time. During first 2 h the decomposition is rather rapid, in the next 4 h it decreases slowly. After 6 h the concentration of diperodon enantiomers in the blood serum does not change and is about 25% for $S(+)$ enantiomer and 33% for $R(-)$ enantiomer. These results may be due to the increasing aniline concentration which is formed from the decomposition of the diperodon enantiomers in the serum. After 2 h the accumulating aniline (an inhibitor of the enzymes responsible for the hydrolysis) caused a decrease in the rate of decomposition of diperodon enantiomers. Similar results for degradation of the racemate of diperodon in blood serum were discussed in literature [15].

The experimental rate constants (k) were determined from the slopes of the linear plots of reciprocal values of the concentration of enantiomeric forms in plasma versus time curves. Table 2 summarises the rate constants and the half-life times of enzymatic hydrolysis for the diperodon enantiomers. A longer half-life for the $R(-)$ enantiomer is indicated. The rate constants for hydrolysis of enantiomers in plasma were different.

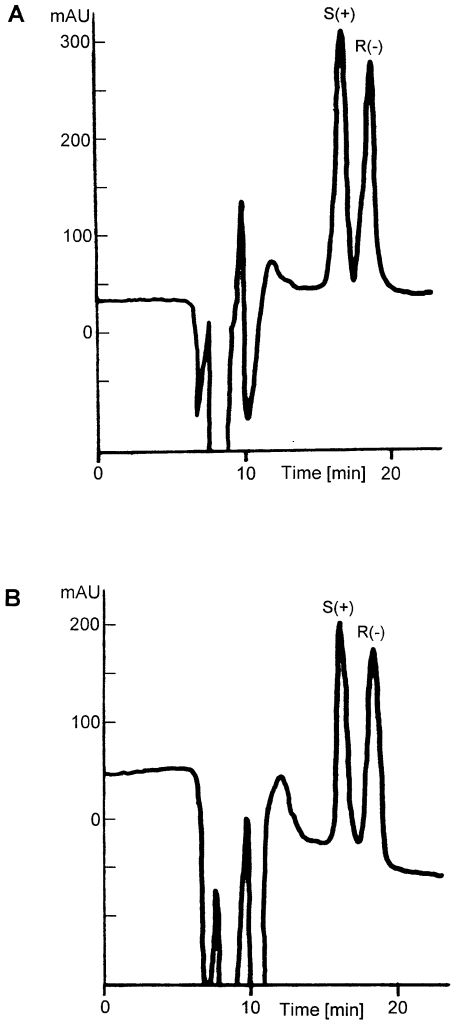

Fig. 4. Chromatograms of the standard solution of diperodon (A) and spiked blood serum (B) obtained with the on-line coupled reversed-phase C18 and teicoplanin chiral stationary phase. Conditions: RP column: methanol/acetonitrile 45/55 v/v; chiral column: methanol/acetonitrile/acetic acid/triethylamine 45/55/0.3/0.2 v/v/v/v; flow rate: 0.5 ml/min; sample: 5 μg/ml of diperodon; injection volume: 50 μl; detection: UV, 240 nm.

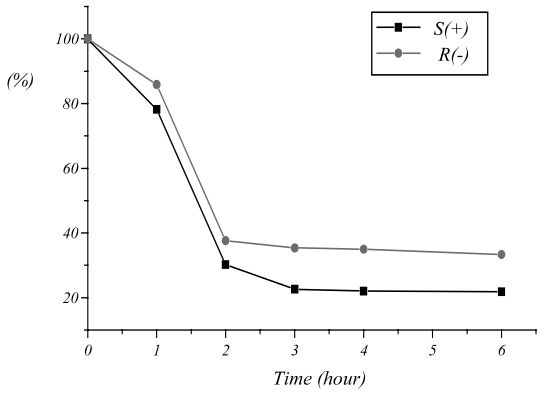

Fig. 5. The time course of the diperodon enantiomers.

Table 2
The rate constants and the half-lives of enzymatic hydrolysis of diperodone enantiomers

	k (mol per 1 s)	$t_{1/2}$ (h)
$S(+)$ enantiomer	14.97 ± 1.35	2.04 ± 0.19
$R(-)$ enantiomer	8.09 ± 0.89	3.36 ± 0.31

References

[1] J.L. Cohen, Anal. Profiles Drug Subst. (London) 6 (1977) 99.
[2] T.H. Rider, J. Am. Chem. Soc. 52 (1930) 2583.
[3] G. Schill, I.W. Wainer, S.A. Barkan, J. Liq. Chromatogr. 9 (1986) 641.
[4] J. Hermansson, A. Grahn, J. Chromatogr. A 694 (1995) 57.
[5] J. Čižmárik, J. Lehotay, K. Hromul'áková, M. Pokorná, M. Lacuška, Pharmazie 52 (1997) 5.
[6] Y. Okamoto, Z.K. Cao, R. Aburatami, K. Hatada, Bull. Chem. Soc. Jpn. 60 (1987) 3999.
[7] N. Ôi, M. Nagase, T. Doi, J. Chromatogr. 257 (1983) 111.
[8] N. Ôi, H. Kitahara, J. Chromatogr. 265 (1983) 117.
[9] D.W. Armstrong, J.T. Lee, L.W. Chang, Tetrahedron Assym. 9 (1998) 2043.
[10] S. Ahuja (Ed.), Chiral Separations Applications and Technology, American Chemical Society, Washington, DC, 1997.
[11] M.J. Michelson, Comparative Pharmacology, vol. II, Pergamon Press, Oxford, New York, Toronto, 1973.
[12] J. Lehotay, K. Hroboňová, J. Čizmárik, M. Renčová, D.W. Armstrong, J. Liq. Chromatogr. Rel. Technol. 24/5 (2001) 609.
[13] S.C. Chang, G.L. Reid, S. Chen, C.D. Chang, D.A. Armstrong, Trends Anal. Chem. 12 (1993) 144.
[14] D.A. Armstrong, S. Chen, C.D. Chang, J. Liq. Chromatogr. 15 (1992) 545.
[15] J. Čizmárik, J. Lehotay, K. Hromul'áková, Acta Polon. Pharm. Drug Res. 53 (1996) 167.