Department of Analytical Chemistry[1], Slovak Technical University, Bratislava, Slovakia, Institut of Pharmaceutical Chemistry[2], University of Muenster, Muenster, Germany

Chiral separation of verapamil and some of its metabolites by HPLC and CE

E. BRANDŠTETEROVÁ[1], G. ENDRESZ[2] and G. BLASCHKE[2]

HPLC and CE assays were developed for chiral separations of verapamil and its metabolites in serum samples. Three chiral HPLC columns (Chiralcel OJ, Chiralpak AD and Chiralcel OD-R) were tested in normal and reverse-phase modes. All HPLC analyses were performed with fluorescence detection at 276 and 310 nm. CE was realized using CM-β-CD as a chiral selector for the enantiomeric analysis. The results of HPLC and CE studies were compared and the possibilities for the applications in therapeutic drug monitoring were discussed.

1. Introduction

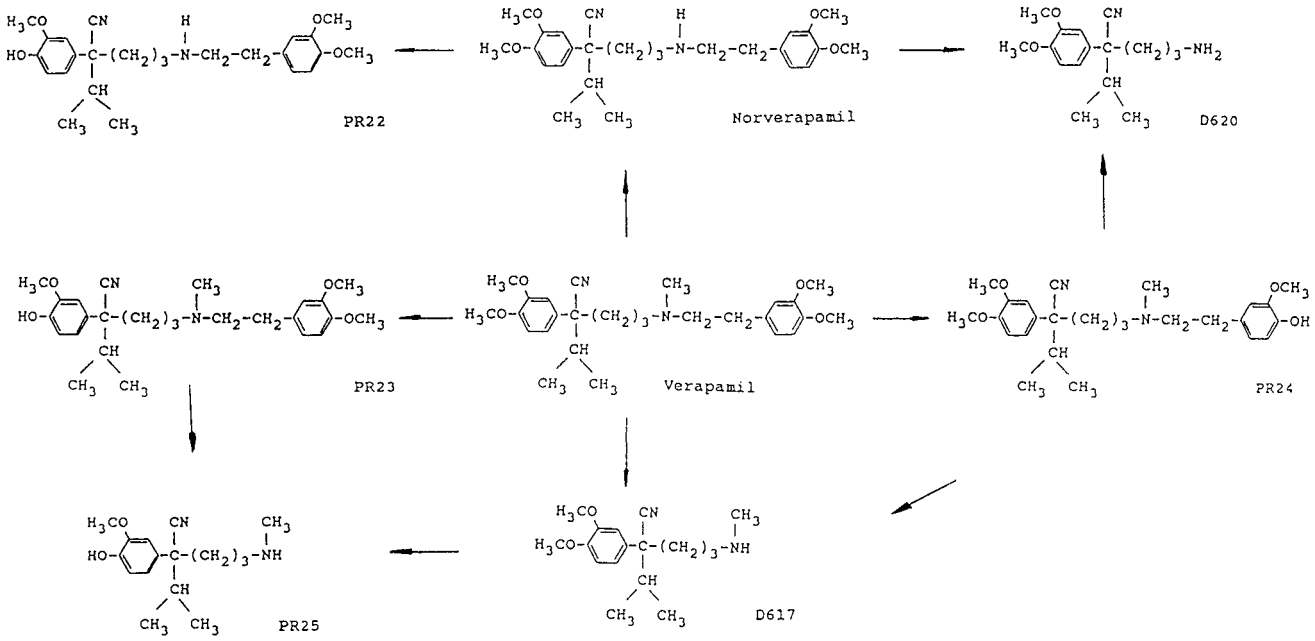
High performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are very important instrumental methods commonly used for the analyses of drugs and their metabolites in clinical samples.

Verapamil (VER) is a calcium channel blocking agent widely applied in the treatment of angina pectoris, supraventricular tachycardia, atrial fibrilation and hypertension. It is extensively metabolized (N-dealkylation, O-demethylation), (Scheme). The major metabolite of verapamil, norverapamil (NOR) posseses a pharmacological activity which is approximately 20% of the activity of the parent compound [1]. Verapamil is a chiral drug and its R(+) and S(−) enantiomers differ in pharmacokinetic and pharmacodynamic properties significantly. The less cardiotoxic compound R(+) verapamil is more than 10 times less potent than S(−) verapamil. Also the protein binding is enantioselective with a greater free fraction of S(−) verapamil compared to R(+) verapamil [2, 3].The enantiomers differ also in their metabolism, toxicity, plasma disposition and urine excretion kinetics [4].

Verapamil is a possible modifier of multi-drug resistance (MDR). *In vitro* experiments have confirmed its activity in increasing cytotoxicity of vinca alkaloids, anthracycline drugs [5] or adriamycin and vinblastin [6, 7]. However it has also been found that high doses of verapamil show cardiotoxicity *in vitro* and it was recommended to apply less cardiotoxic calcium channel blockers, that could be administered in higher concentrations. R(+) verapamil is less cardiotoxic but has equivalent activity in MDR. Moreover, norverapamil is also effective in reversing MDR. This major verapamil metabolite is a chiral compound and the application of less toxic R(+) norverapamil in MDR could be taken into the consideration [8].

As the other metabolites of verapamil are chiral too, it could be very interesting to develop an assay for their chiral separation in clinical samples to have more information about the biotransformation pathway. The simultaneous achiral separation of verapamil and its seven metabolites in combination with off-line and on-line SPE has been published previously [9]. The complete SPE-HPLC assay of all compounds was performed in about 40 minutes.

Scheme



The chiral separations of verapamil and norverapamil have already been published [10, 11] but the other verapamil metabolites were not taken into consideration in these papers. Chu and Wainer [10] reported the first achiral-chiral separation of verapamil and norverapamil in serum samples. Fieger and Blaschke [11] published two methods for the determination of the enantiomeriic ratio of verapamil in plasma using an AGP column. Verapamil was separated after acetylation of norverapamil. On an amylose chiral column (Chiralpak AD) verapamil and norverapamil were determined simultaneously without prior derivatization [11]. The stereochemical composition of verapamil and some of its metabolites in dog urine samples was determined using an ovomucoid chiral column, but no simultaneous chiral separation of verapamil and its metabolites was achieved [12].

The chiral separation of verapamil and its seven metabolites using an AGP column has also been published previously [9]. As it was observed, only some of the metabolites could be separated simultaneously. In some cases two or three compounds were analyzed in one run. The simultaneous chiral separation of norverapamil and its two metabolites in serum and microsomal samples was recently demonstrated [13].

Capillary electrophoresis (CE) has also been applied in a chiral analysis of verapamil and norverapamil. Cyclodextrins (CDs) are the most commonly used chiral selectors in CE which were the first time described by Fanali [14]. Besides the native CDs, derivatized uncharged and charged CDs become popular as potential chiral selectors in CE [15–18]. Chiral separation of some basic drugs including verapamil in serum by CE with modified CD buffers has also been presented [19]. In this study, cationic detergents were used in the mixed-micellar mode. Dethy et al. achieved a simultaneous separation of verapamil and norverapamil enantiomers with trimethyl-β-CD(TM-β-CD), an uncharged CD derivate [20]. Chiral recognition of verapamil by various CDs has also been studied [21] using CE, NMR spectroscopy and ESI-MS spectroscopy.

The aim of this work was the comparison of HPLC and CE simultaneous chiral separations of verapamil, norverapamil and some of the other verapamil metabolites. In the HPLC assay different chiral stationary phases and mobile phases were tested. SPE has been used as a preseparation step before the chiral analyses of serum samples. For the CE chiral separation, the use of carboxymethyl-β-CD (CM-β-CD) as a chiral modifier was studied.

2. Investigations, results and discussion

In our previous papers [9, 13], the HPLC chiral separations of verapamil and its seven metabolites were performed using an AGP column and water-organic mobile phase. All the studied metabolites were separated but the simultaneous chiral separation of verapamil and its metabolites was not always possible, so the indirect chiral separation of fractions from achiral column or the achiral-chiral column-switching system had to be used, a procedure which is relatively complicated in routine clinical studies. Moreover, the on-line SPE procedure, applied for achiral HPLC separation, could not been simply be incorporated into the achiral-chiral column switching system. For these reasons, the possibility of a simple simultaneous chiral separation of verapamil, norverpamil and their main metabolites has been studied in this work. Both HPLC and CE techniques were used and the results are compared.

Capillary electrophoresis has been applied for simultaneous chiral separation of verapamil and some of its metabolites using carboxymethyl-β-CD (CM-β-CD) as a chiral buffer modifier. As it is obvious from the Table, the enantioseparation of verapamil and its metabolites was achieved using 5 mM CM-β-CD. Gallopamil could be used as an internal standard for the assay. PR 23, PR 24 and PR 25 could not be chirally separated in one run, as the differences in their retention times were very small. Individual chiral separations of verapamil and its metabolites are presented in Fig. 1. The simultaneous CE chiral separation was achieved for verapamil, norverapamil and their main metabolites, D 617 and D 620, (Fig. 2). This was not possible using an HPLC chiral column [9]. Fig. 3 confirms the fact, that the separation order for R(+) and

Table: Migration times of verapamil and its metabolites

Compound	t_{m1} (min)	t_{m2} (min)	α_{rel}
Verapamil	21.8	22.5	1.03
Norverapamil	22.1	22.9	1.04
D 620	16.1	17.2	1.07
D 617	16.9	17.9	1.06
PR 22	20.3	20.8	1.02
PR 23	19.7	20.0	1.02
PR 24	19.7	20.1	1.04
PR 25	18.6	19.5	1.04
Gallopamil	12.2	12.3	1.01

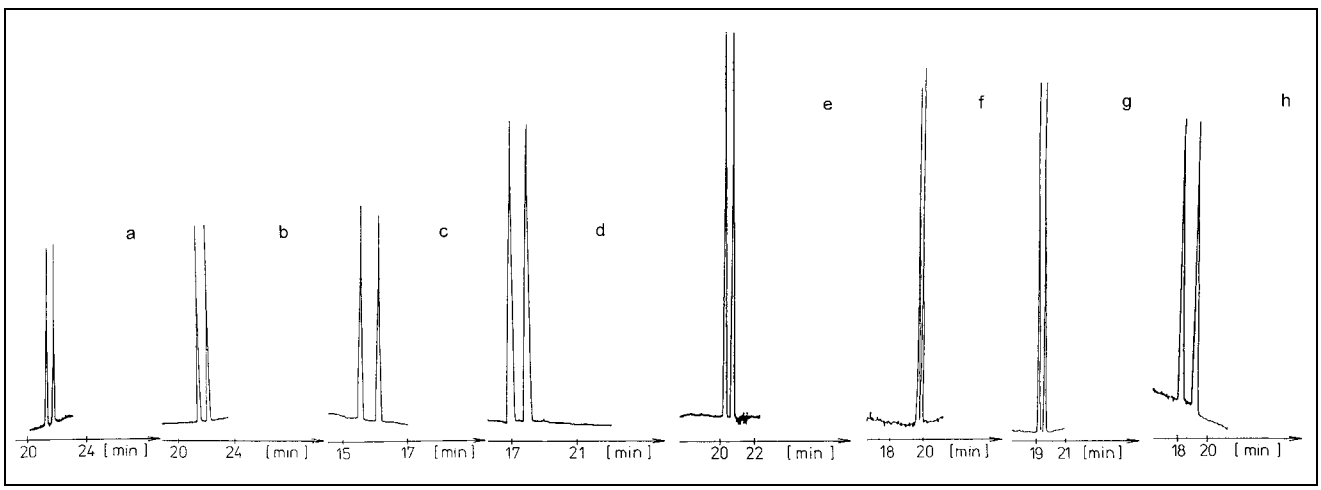


Fig. 1: CE chiral separation of verapamil and its metabolites. (a) verapamil, (b) norverapamil, (c) D 620, (d) D 617, (e) PR 22, (f) PR 23, (g) PR 24, (h) PR 25. Conditions: 5 mM CM-β-CD, 50 mM phosphate buffer, pH = 3, detection 210 nm, 400 V/cm

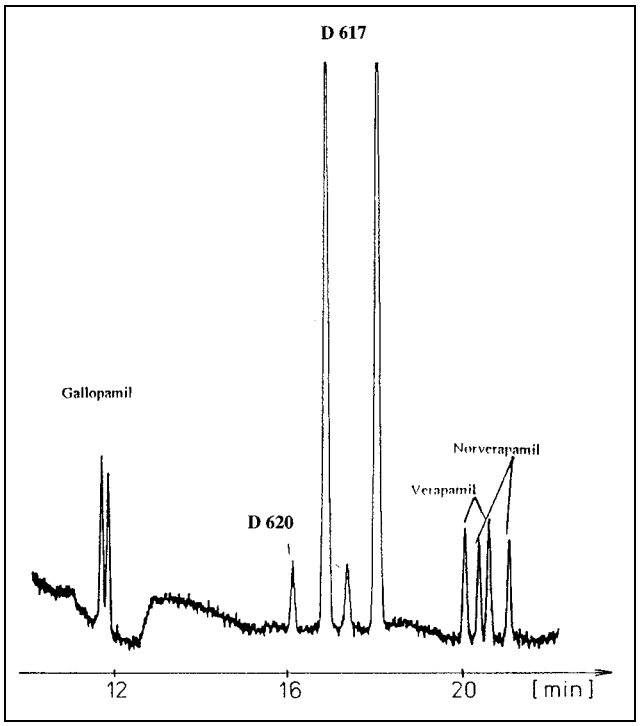


Fig. 2: CE chiral separation of verapamil, norverapamil, D 617 and D 620. Conditions: as in Fig. 1

S(−) enantiomers of verapamil and norverapamil using CM-β-CD is different with the use of TM-β-CD [20]. The S(−) enantiomer is eluting before the R(+) one. Both cyclodextrins form inclusion complexes with enantiomers and the stronger binding enantiomer or enantiomers staying longer in the CD cavity, will migrate as second peaks. Thus, reversal enantiomer migration was observed with CM-β-CD, which means that both CDs have a different chiral recognition to verapamil and its metabolites. The reversal separation sequence in our case has an important advantage for the determination of the enantiomeric purity, as the first eluting S(−) enantiomer is often present in very low concentrations. According to the differences in structures of all analysed compounds, it is possible to divide them into two groups. The compounds in the first group, (verapamil norverapamil, PR 22, PR 23 and PR 24) have two aromatic rings after metabolism, their retention times are longer in CE and HPLC. The second group (PR 25, D 617 and D 620) has only one aromatic ring and this is a reason for the lower retention of analytes and smaller interactions with the chiral stationary phases in HPLC or chiral modifiers in CE. The small differences in retention behaviour of verapamil and norverapamil are caused by the fact, that these compounds differ only in a methyl group. This fact makes the simultaneous separation more difficult. We find the same situation for PR 22 and PR 23 and PR 23 and PR 24 where only the position of the hydroxy group is different.

HPLC chiral separation of verapamil and its main metabolites has been performed with three different chiral columns, Chiralcel OJ, Chiralpak AD and Chiralcel OD-R. According to the Daicel information all of them are recommended for the chiral separation of compounds with aromatic, cyano and hydroxyl groups. The last column was working in a reversed-phase mode, OJ and AD columns have been applied in a normal mode with organic mobile phases. The Chiralcel OJ cellulose ester derivate column was tested using a mobile phase consisting of ethanol, iso-propanol in n-hexane without or with the addition of 0.5% of triethylamine (TEA), at a flow-rate of 1ml/min. The chiral separation of verapamil, norverapamil and PR 22 on an OJ column is shown in Fig. 4. The mobile phase consisted of 7.5% ethanol, 7.5% iso-propanol and 85% n-hexane. Obviously, this column does not allow the simultaneous separation of verapamil metabolites. Verapamil, norverapamil and compounds with two aromatic rings (PR 22, PR 23 and PR24) were separated too, but the group of metabolites with one aromatic ring (PR 25, D 617 and D 620) were not eluted from the OJ column at all as they are too polar for the elution by a mobile phase with so high content of non polar organic solvent (n-hexane). The addition of TEA to the mobile phase increases the chromatographic resolution of enantiomers but also retention times become longer (Fig. 5). It is obvious that this column is not able to separate verapamil metabolites simultaneously.

Chiralpak AD amylose derivate columns have also been recommended for the analysis in a normal mode. For this chiral column, a mobile phase consisting of ethanol and n-hexane (1 : 9) was chosen with flow rates of 0.9 ml/min and 1 ml/min. The chiral separation was successful for all the two aromatic ring compounds using an OJ column in

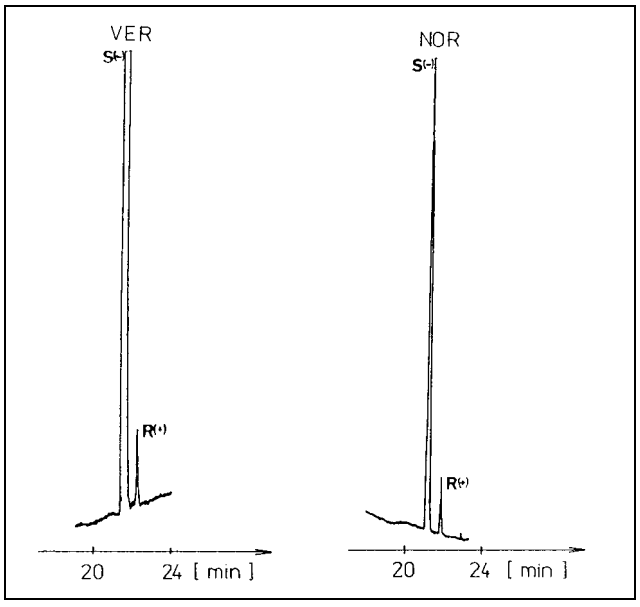


Fig. 3: Elution order of enatiomers (a) verapamil, (b) norverapamil. Conditions: as in Fig. 1

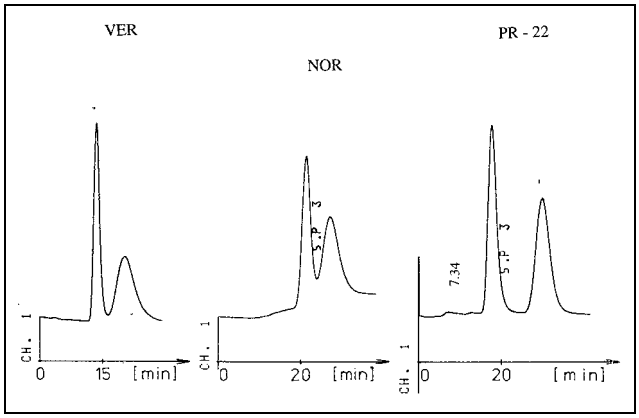


Fig. 4: HPLC separation of verapamil and its metabolites on OJ column (a) verapamil, (b) norverapamil, (c) PR 22. Mobile phase: 85% n-hexane, 7.5% ethanol, 7.5% iso-propanol, flow-rate 1 ml/min

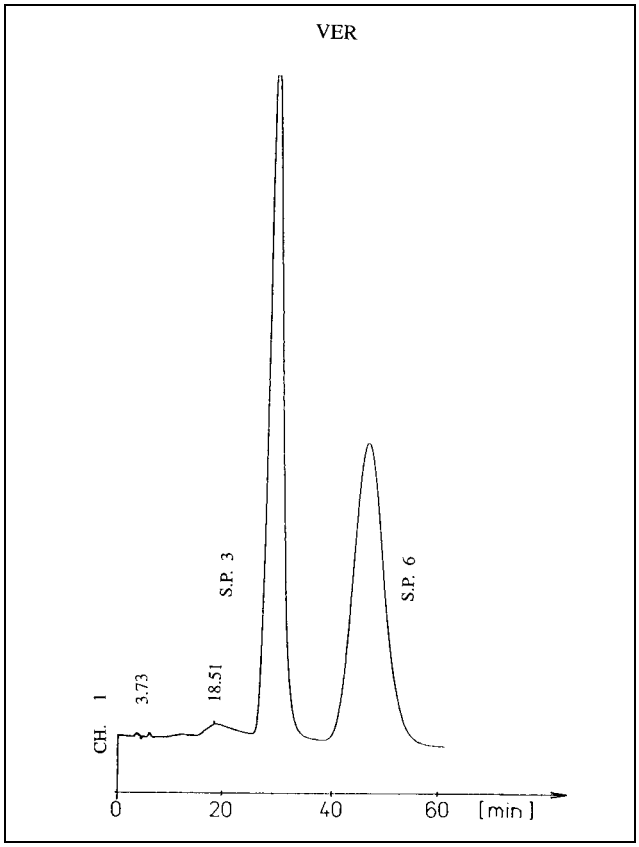


Fig. 5: HPLC chiral separation of verapamil on OJ column with TEA. Conditions: Chiralcel OJ column, 90% n-hexane, 10% ethanol, 0.5% TEA, flow-rate 1 ml/min

a normal mode. The AD column enabled the simultaneous separation of verapamil and norverapamil, with one of the metabolites from two aromatic ring components (PR 22, PR 23 or PR 24) additionally. One aromatic ring compounds have not been eluted from the column in a reasonable analysis time. This fact could again be explained by high retention of these relatively polar compounds at the chiral column and low affinity to the mobile phase with a small content of polar modifiers. The chiral HPLC separation of a mixture of verapamil, norverapamil and PR 22 on the AD column is shown in Fig. 6.

The next column (Chiralcel OD-R) was developed for application in the reverse-phase mode. The following mobile phases have been tested: acetonitrile in 0.2 M $NaClO_4$ (40 : 60) and acetonitrile in 0.1 M KPF_6 (40 : 60), at a flow-rate of 0.7 ml/min. The results confirmed the data obtained with an AGP chiral column [9], which means that verapamil norverapamil, PR 22, PR 23 and PR24 were separated. However, their simultaneous separation was not possible due to the reasons discussed above. Moreover, one ring metabolites (PR 25, D 617 and D 620) were eluted from the column in very short elution times not separated, as it possible to see in Fig 7.

Summarizing results from CE and HPLC chiral analyses of verapamil and its metabolites, it is possible to observe that CE enables the separation of all the compounds studied into both enantiomers, but only verapamil, norverapamil, D 617 and D 620 could be separated in one run. The next separations could be achieved for verapamil, norverapamil and the other two ring compounds (PR 22, PR 23 or PR 24) instead of D 617 and D 620. It means, maximum four analytes have been separated simultaneously. HPLC chiral analysis of verapamil and all its metabolites has

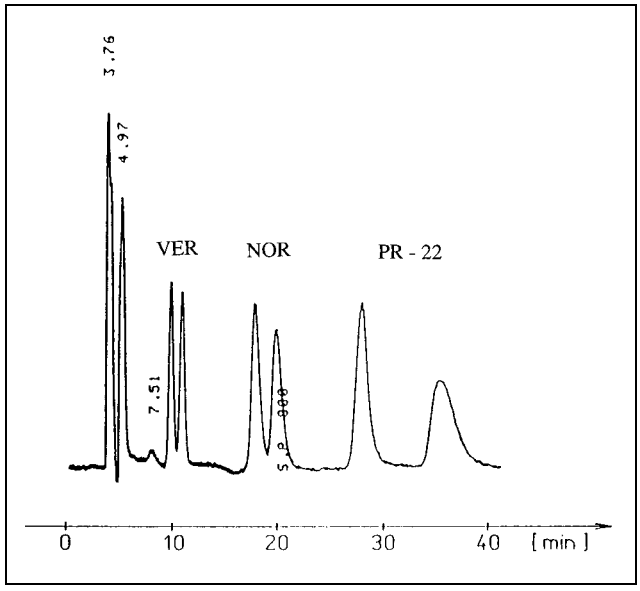


Fig. 6: HPLC separation of verapamil, norverapamil and PR 22. Conditions: Chiralpak AD column, 90% n-hexane, 10% ethanol, flow-rate 0.7 ml/min

only been achieved on an AGP column [9] what is influenced by the high binding affinity of basic of verapamil and its metabolites to a chiral column with an acidic character.

The OD-R column, which also worked in the reversed-phase mode as an AGP column, allows the chiral separation of all two ring compounds but they could also not be separated in one run. The next two columns tested, OJ and AD columns were applied in a normal mode and only AD column has given the possibility to separate three compounds in one run (verapamil, norverapamil and one metabolite with two rings, e.g. PR 22 (Fig. 6). For this reason, some clinical samples of patients from the National Cancer Institute, Bratislava, Slovakia were separated after an off-line SPE preseparation step [9] using an AD column. Verapamil and norverapamil were separated and the ratio of their enantiomers were determined. These results will be published later. HPLC chiral separation of the serum sample on an AD column is illustrated in Fig. 8a, b. The chromatogram of blank patient serum after SPE, before verapamil application is shown in Fig. 8a. The advantage of an AD column application is the fact, that the separation sequence of enantiomers is reverse in comparison to AGP and OD-R columns. This means, that the separation order is the same as in the CE assay, which what is very useful in cases when very low concentrations of S(−) enantiomers are present in clinical samples.

3. Experimental

3.1. Equipment and conditions

The HPLC pump Beckman, model 110A, a six-port Rheodyne 7125 injection valve and a RF 535 fluorescence detector Shimadzu were used for chiral HPLC analyses and chiral columns Chiracel OD-R, Chiracel OJ and Chiralpak AD, (46 × 250 mm), Daicel, Duesseldorf, Germany were tested.

The mobile phases tested for chiral separations were: CH_3CN and $NaClO_4$ and CH_3CN and KPF_6 for OD-R, iso-propanol and ethanol in n-hexane for OJ and ethanol in n-hexane for AD columns.

A Grom capillary electrophoresis system 100 (Herrenberg, Germany), equipped with a Linear Instruments (Reno NV, USA), UV-VIS 200 detector were used with an untreated fused-silica capillary (Grom) of 61 cm total length and 50 μm I.D. Samples were introduced hydrostatically

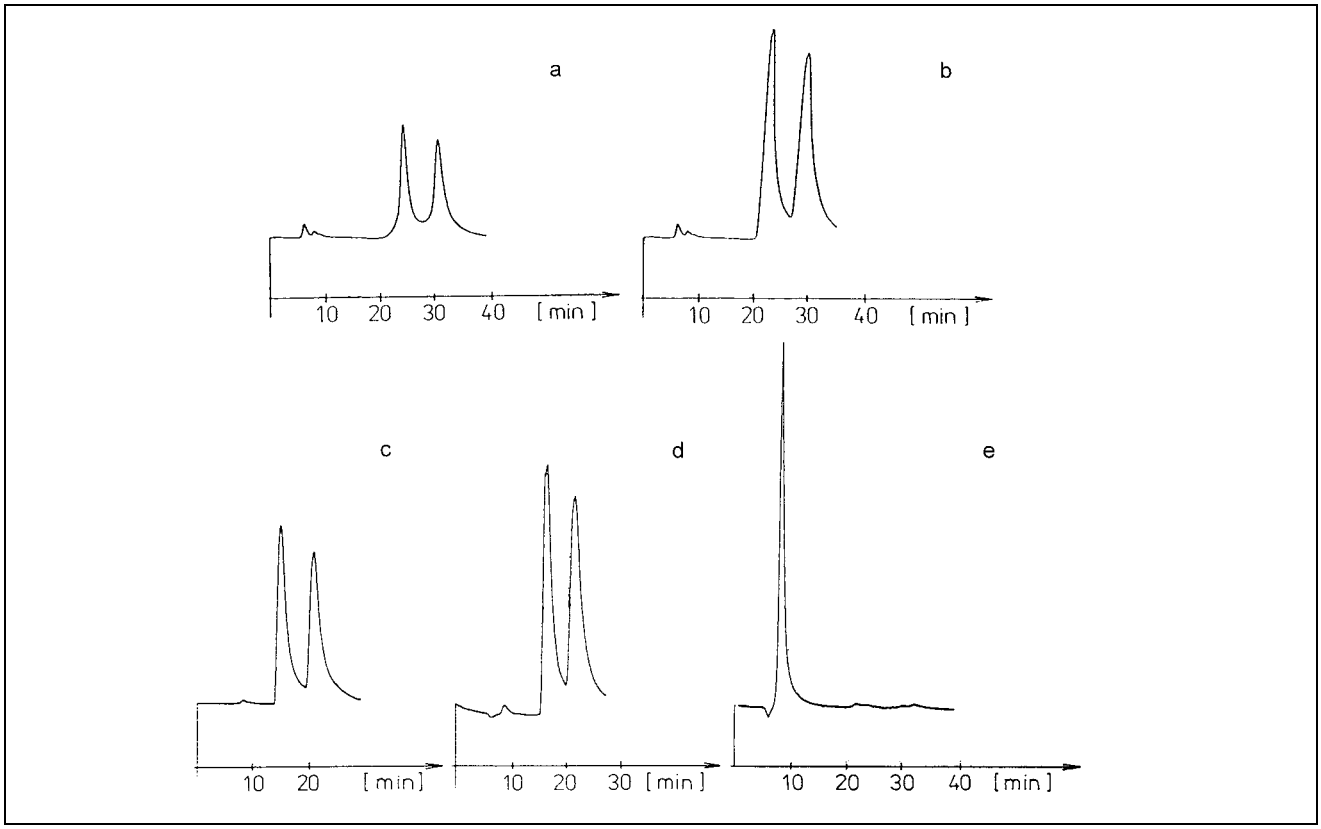


Fig. 7: HPLC separation of verapamil and its metabolites using Chiralcel OD-R column (a) verapamil, (b) norverapamil, (c) PR 22, (d) PR 24, (e) D 620. Mobile phase: 40% acetonitrile, 60% KPF_6, flow-rate: 0.7 ml/min

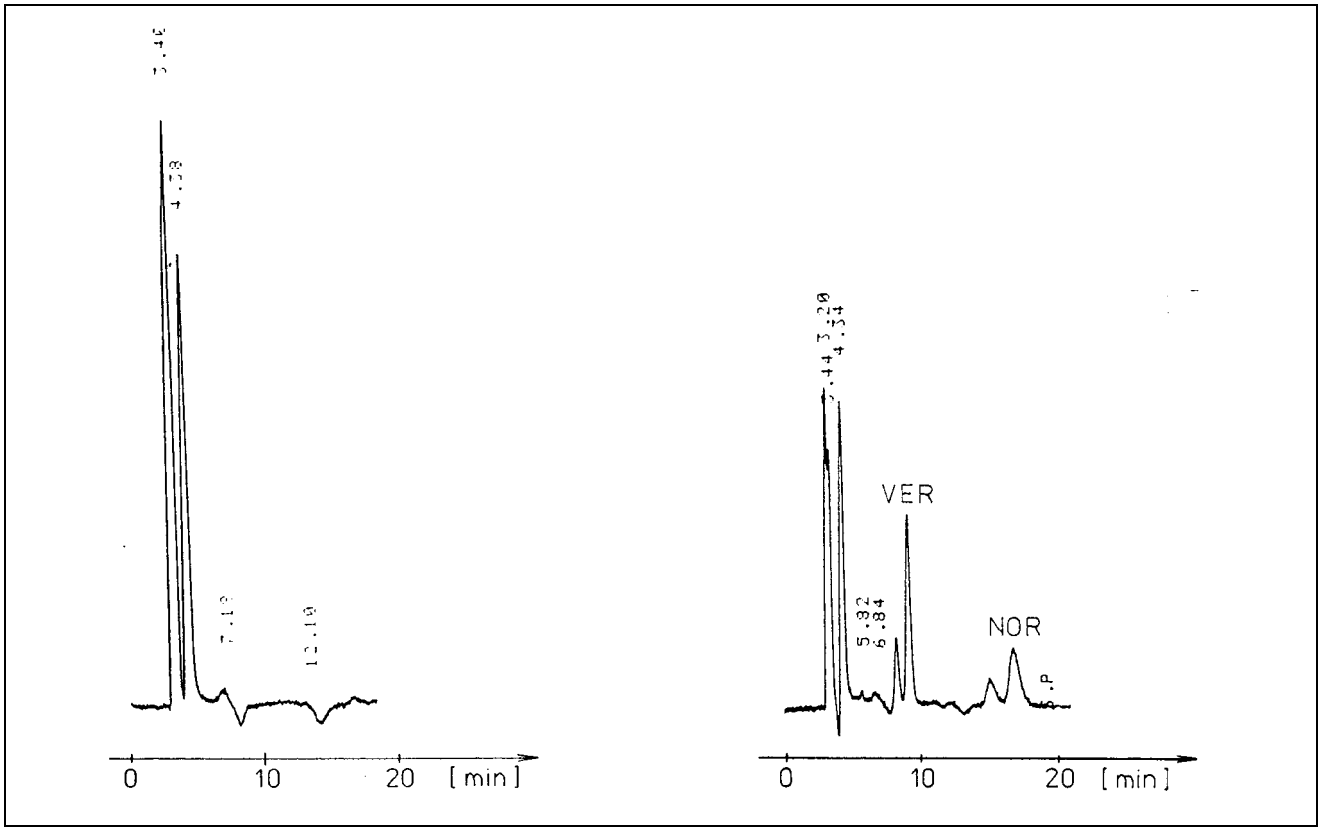


Fig. 8: HPLC separation of SPE serum extract on Chiralpak AD column (a) blank, (b) serum extract. Conditions: as in Fig. 6

(10 cm) during 5 s at the anodic end of the capillary. The detection was performed at 210 nm and the electric field was +400 v/cm, temperature 21 ± 1 °C.

3.2. Chemicals

Standards of verapamil hydrochloride, norverapamil hydrochloride, verapamil R-(+) enantiomer, verapamil S-(−) enantiomer, norverapamil R-(+) enantiomer, norverapamil S-(−) enantiomer, D 617, D-620, PR 22, PR 23, PR 24 and PR 25 were kindly supplied by Knoll, Ludwigshafen, Germany. Methanol, acetonitrile (gradient grade), n-hexane, ethanol, iso-propanol, triethylamine, phosphoric acid, dihydrogen calium phosphate and disodium hydrogen phosphate (analytical grade) were purchased from Merck, Darmstadt, Germany. Carboxymethyl-β-CD was from Wacker Chemie, Munich, Germany.

3.3. Solution and sample preparation

Stock solutions of standards of verapamil and its metabolites were prepared in concentrations of 1 mg/10 ml in methanol and the working solutions were prepared daily diluting stock solutions with water.
Stock solutions of 50 mM KH_2PO_4 for CE analyses were prepared in double distilled, deionized water. The pH was adjusted with 0.5 M phosphoric acid (pH = 3). The run buffer was prepared after the addition of appropriate amounts of the chiral selector (5 mM CM-β-CD) as a leading buffer. The same phosphate buffer was used as an ending buffer. All solutions were filtered and degassed before analysis.
A SPE procedure clean-up step was realized as previously described [9, 13]. SepPak C-18 cartridges (Waters, Milford USA) were washed with 3 ml of methanol, 3 ml water. 0.5 ml of serum diluted with 0.5 ml of water was loaded on the cartridge and the analytes were, after a washing step with 2 ml water, eluted with 1 ml of methanol with 0.3% TEA. The eluate was evaporated to dryness in vacuo and the residue dissolved in the mobile phase was injected into the HPLC or CE systems.

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Assoc. Prof. Dr. Eva Brandšteterová, PhD
Department of Analytical Chemistry
Slovak Technical University
Radlinského 9
812 37 Bratislava
Slovakia
branstet@cvt.suba.sk