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Development and validation of a bioanalytical method for the quantification of diltiazem and desacetyldiltiazem in plasma by capillary zone electrophoresis

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Abstract

A CZE method for the quantification of diltiazem and desacetydiltiazem in plasma was developed and validated. Separation was accomplished at pH 2.5 in a 0.044 *M* phosphate buffer. Sample preparation was performed by liquid–liquid extraction and no interferences with plasma compounds were detected. The calibration graph is linear over the range 5–250 ng/ml with verapamil as internal standard. The precision and accuracy are better than 13% at 5 ng/ml, and better than 10% between 10 and 250 ng/ml. The long-term reliability of the CZE system was checked over a 3-month period. The CZE method is a useful alternative to the already established HPLC method.

1. Introduction

Diltiazem (DTZ) (Fig. 1) is a calcium channel blocker used in the treatment of angina pectoris, hypertension and supraventricular tachyarrythmias [1]. Several high-performance liquid chromatographic (HPLC) methods have been developed for the determination of diltiazem and the major metabolite desacetyldiltiazem (M1) [1,2] in plasma. The methods described usually involve an extraction step for sample clean-up and concentration.

The main analytical tools for monitoring drugs in body fluids in pharmacokinetic studies are HPLC, GC and immunoassays. Each of these techniques, however, has certain restrictions. The use of capillary zone electrophoresis (CZE) or micellar electrokinetic chromatography (MEKC) in that field is not very common, and only a few reports have appeared [3]. One reason for this may be the lower concentration sensitivity of CE systems in comparison with HPLC [4]. Different attempts have been made to enhance the sensitivity in CZE by improving the detection techniques and employing sample pretreatment prior to the CZE separation [5].

The separation and determination of DTZ and its metabolites by MEKC was investigated by Nishi et al. [6–8]. To our knowledge, no quantitative bioanalytical methods using CZE or MEKC have been published. In this paper, we describe the method development and validation of a bioanalytical method and demonstrate the suitability of CZE as an alternative to HPLC for pharmacokinetic studies with drug concentrations in the low ng/ml range.

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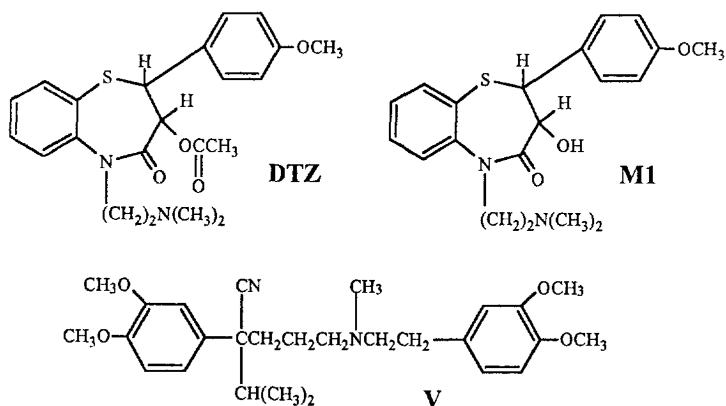

Fig. 1. Structures of diltiazem (DTZ), desacetyldiltiazem (M1) and verapamil (V).

2. Experimental

2.1. Instrumental

CZE system

CZE experiments were carried out on an HP^{3D} CE– system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode-array detector. Capillaries with 50 μm I.D. and 365 μm O.D., standard detection window and bubble cell (extended light path) were obtained from Hewlett-Packard. All capillaries had a total length of 64.5 cm and an effective length of 56 cm. Data processing was done on a Hewlett-Packard Vectra 486/66 XM computer using the HP^{3D} CE – software for instrument control, signal integration and spectral analysis. Statistical calculations were performed with Microsoft EXCEL 5.0 software.

HPLC system

The chromatographic system consisted of a Spectra-Physics (San Jose, CA, USA) SP 8810 double-piston pump, an SP 8780 autosampler (injection volume 50 μl), an SP 8490 UV detector (set to 238 nm) and an SP 4270 integrator. Chromatographic experiments were performed on a Spherisorb ODS 2 (10 μm) column (250 × 4 mm I.D.) (Promochem, Wesel, Germany). The mobile phase was acetonitrile–0.01 *M* ammonium phosphate buffer (aqueous solution of 1.15 g/l $NH_4H_2PO_4$ plus 0.6 ml of triethylamine, adjusted to pH 3.7 with phosphoric acid) (60:40).

2.2. Materials

Water was deionized with a Milli-Q Reagent Grade Water System (Millipore, Molsheim, France). Phosphoric acid extra pure (85%), sodium dihydrogenphosphate dihydrate extra pure and tert.-butyl methyl ether for residue analysis were purchased from Merck (Darmstadt, Germany). Methanol (HPLC grade), acetonitrile and triethylamine (analytical-reagent grade) were obtained from J.T. Baker (Deventer, Netherlands). During method development, the following 20 m*M* buffer solutions for HPCE from Fluka (Buchs, Switzerland) were used: sodium citrate buffers of pH 3.0, 4.5 and 6.0 and sodium phosphate buffers of pH 6.5 and 7.5. Sodium dihydrogenphosphate buffer solutions in the pH range 2.0–2.5 and with concentrations between 0.020 and 0.088 *M* were freshly prepared for the separation experiments by dissolving $NaH_2PO_4 \cdot 2H_2O$ in deionized water and adjusting the pH with phosphoric acid (85%).

Diltiazem hydrochloride and verapamil hydrochloride were supplied by Welding (Hamburg, Germany). Desacetyldiltiazem was synthesized

by ester hydrolysis from diltiazem and purified and characterized according to the literature.

2.3. Procedures

Capillary washing was executed with 1 and 0.1 *M* sodium hydroxide solutions for HPCE (Fluka) and with buffer solution. New capillaries were preconditioned by rinsing for 10 min with 1 *M* NaOH, 5 min with 0.1 *M* NaOH and 5 min with buffer solution. Before each analysis, the capillary was purged for 1.5 min with 0.1 *M* NaOH and 3 min with buffer.

Electrokinetic injection was tested for aqueous solutions of diltiazem hydrochloride and desacetyldiltiazem in the voltage range 0.75–10 kV and time range 1–10 s. Solutions containing 0.017 *M* phosphoric acid were checked with injection voltages ranging from 2.6 to 30 kV. The injection time was 10, 15 or 20 s.

Hydrodynamic injection was performed by applying a pressure of 50 mbar to the sample. The injection time was varied between 20 and 150 s. After hydrodynamic sample injection, buffer solution was injected for 20 s at 50 mbar.

2.4. Standard solutions

DTZ and M1

Stock standard solutions of the appropriate amount of diltiazem hydrochloride, corresponding to 1 mg/ml diltiazem free base and 1 mg/ml desacetyldiltiazem free base, were prepared in water and methanol, respectively. Aliquots were subsequently diluted with water to obtain 10 and 1 μg/ml working standard solutions.

Internal standard

A 1 mg/ml aqueous solution of verapamil hydrochloride was prepared and further diluted with water to give working standard solutions of 2.5 μg/ml for the CZE method and 0.5 μg/ml for the HPLC method. A 250-μl aliquot (625 ng for the CZE method and 125 ng for the HPLC method) was added to each 1-ml aliquot of plasma standard or specimen.

2.5. Extraction procedure

To a 100 × 13 mm glass tube, fitted with a glass stopper, were added 1 ml of plasma, 250 μl of internal standard solution and 5 ml of tert.-butyl methyl ether as extraction solvent. The tubes were shaken for 10 min on a Reax2 overhead mixer (Heidolph, Kelheim, Germany) and then centrifuged at 1800 *g* for 10 min. A 4-ml aliquot of the organic phase was transferred into a glass tube and back-extracted with 40 μl (CZE method) or 150 μl (HPLC method) of 0.017 *M* phosphoric acid by shaking for 3 min on an overhead mixer. After centrifugation at 2800 *g* for 10 min, the organic phase was discarded and the phosphoric acid solution was transferred into a vial and an aliquot of ca. 45 nl (CZE method) or 50 μl (HPLC method) was injected.

2.6. Calibration

Calibration graphs were constructed by transferring aliquots of the respective 1 μg/ml standard solutions to blank plasma to give final concentrations of 5, 10, 25, 50, 100 and 250 ng/ml. These calibration standards were extracted as described above. The concentrations of DTZ and M1 in samples were determined by using linear regression (1/concentration weighted) of peak area ratios.

3. Results and discussion

As a starting point for CE method development, we used our already existing HPLC method used previously for the pharmacokinetic characterization of a diltiazem retard formulation. This HPLC method includes two liquid–liquid extraction steps and uses the calcium channel blocker verapamil (V) as internal standard (I.S.). This HPLC method is mainly based on the system described by Dubé et al. [9].

Nishi et al. [6–8] reported the separation of DTZ and metabolites by MEKC; no separation was achieved in CZE systems in the pH range 7–9.

To obtain a reliable CZE method for the

quantification of DTZ and M1 in plasma, overcoming the lack of concentration sensitivity of CE, the bioanalytical method was optimized with respect to the separation and quantification of DTZ, M1 and internal standard verapamil (V); short- and long-term testing of the reproducibility and reliability of the CZE system and method; sample preparation of concentration of the analytes and removal of the interfering plasma compounds; and validation of the CZE method.

3.1. Separation and quantification

Experiments with DTZ, M1 and IS in aqueous solutions

To establish the optimum conditions for separation and quantification, we used an aqueous solution of 10 μg/ml each of DTZ, M1 and internal standard verapamil. We investigated separation buffers between pH 2.0 and 9.0. Decreasing the buffer pH below 4.5 resulted in complete separation of DTZ and M1. The best results were obtained at pH 2.5 with a 0.044 *M* phosphate buffer.

We studied the effects of different modes of run control, different injection modes and different procedures for capillary washing and their influence on the relative standard deviation (R.S.D.) ($n=6$) of the peak-area values of DTZ and M1. Our results can be summarized as follows: (1) there was no significant difference between power, current and voltage control; (2) hydrodynamic injection is more reliable than electrokinetic injection; and (3) washing with 0.1 *M* NaOH and buffer before each run was necessary.

With this preliminary method, we started the next optimization cycle. We sought the best compromise between increased sample loading and better signal-to-noise ratio without losing too much resolution and plate number. Performance data were determined for injection times between 20 and 70 s. The best signal-to-noise ratio was achieved at an injection time of about 45 s. The resolution between M1 and DTZ and the number of theoretical plates were maximum at 20 s and minimum at 70 s; the resolution ranged between 4.2 and 0.7 and plate numbers between 207 000 and 15 000. As the best compromise, we selected hydrodynamic injection at 50 mbar for 40 s. The results for DTZ, M1 and IS in aqueous solution are shown in Fig. 2.

Long-term reliability of the CE system

In addition to the optimization procedure, we were interested in the long-term reliability of the CE system. Therefore, we also used an aqueous 10 μg/ml mixture, which was injected six times per sequence. Engelhardt et al. [4] reported that in general the R.S.D. for an external standard method usually ranges between 2 and 3%.

In our long-term testing, over 600 injections were preformed over a period of 3 months. The R.S.D. of each sequence ($n=6$) for the peak-area values was calculated. The R.S.D.s were in the range 0.5–5%, most being in the range 1–

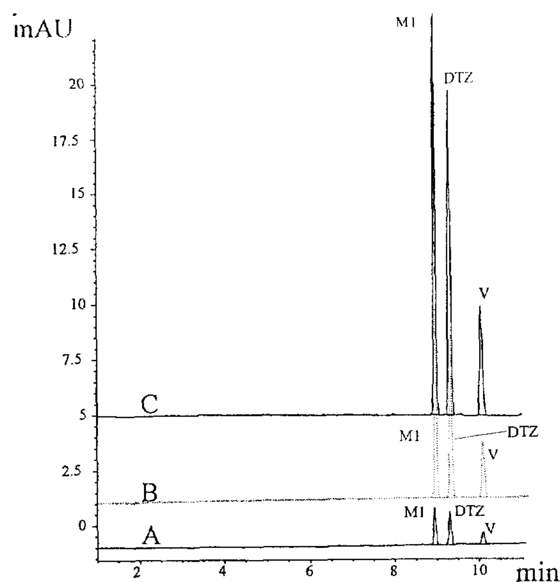

Fig. 2. Electropherograms of three solutions containing desacetyldiltiazem (M1), diltiazem (DTZ) and verapamil (V) in 0.017 *M* phosphoric acid. Each compound has a concentration of (A) 1, (B) 5 and (C) 10 μg/ml. Buffer, 6.9 g/l $NaH_2PO_4 \cdot 2H_2O$ (pH 2.5); capillary, fused silica, 64.5 cm (56 cm to detector) × 50 μm I.D, no extended light path; voltage, 30 kV; detection, 238 nm; temperature, 25°C; injection, hydrodynamic, 50 mbar sample for 40 s followed by 50 mbar buffer for 20 s.

3% (see Fig. 3). No improvement was achieved with migration time-corrected area values.

When using the internal standard method (area for M1 divided by area for internal standard), the R.S.D. of the area ratio was nearly always below 1.5% (Fig. 3). This gives good confidence in the reliability of the CE system, which is essential for working with limited amounts of plasma in pharmacokinetic studies.

3.2. Sample preparation

The problem of establishing a CZE or MEKC method for the determination of DTZ and M1 in

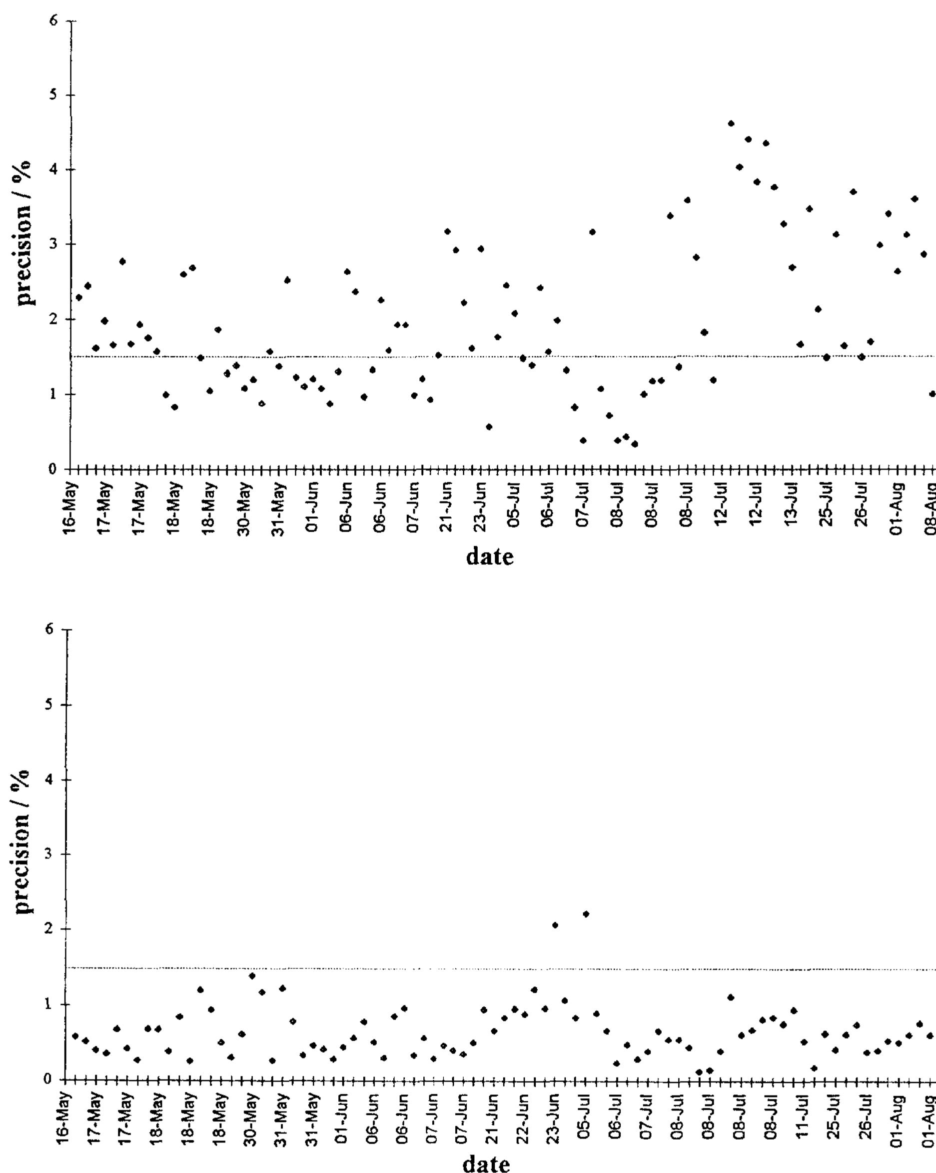

Fig. 3. Temporal variation of the precision of area data for desacetyldiltiazem (top) and area ratio data (bottom). For each sequence of six injections the R.S.D. was calculated.

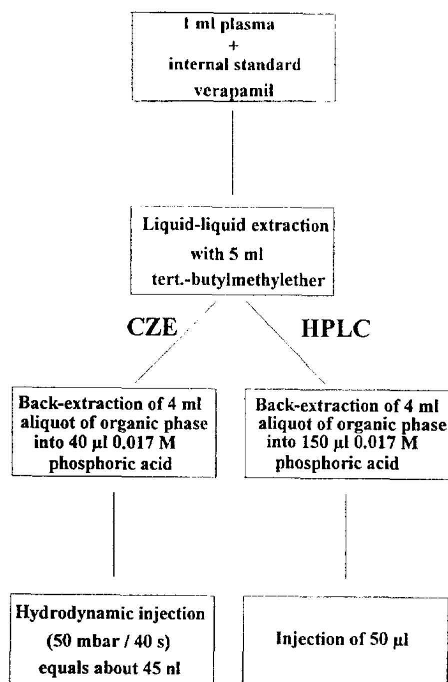

Fig. 4. Sample preparation of CZE and HPLC methods.

plasma is sensitivity. Using an aqueous solution of DTZ and M1, the detection limit (signal-to-noise ratio ca. 3:1) is more than fifteen times higher for the CZE system (ca. 50 ng/ml) than for the HPLC system (ca. 3 ng/ml) (for conditions see Figs. 2 and 6). Calculating the absolute sample loading, based on injection volumes of 45 nl and 100 μl, respectively, the CZE system (2.3 pg) is more sensitive than the HPLC system (300 pg). For a bioanalytical method, the concentration sensitivity is usually more important.

We focused our attempts on determining down to 5 ng/ml levels of DTZ and M1 in plasma by optimizing the sample preparation. Sample preparation can be performed by a variety of methods, mainly liquid–liquid extraction (LLE), liquid–solid extraction (LSE) and protein precipitation [10]. For DTZ and M1 we obtained the best results for the HPLC assay with LLE with tert.-butyl methyl ether. We modified the LLE by back-extraction into 40 μl of 0.017 *M* H_3PO_4, as described in Fig. 4.

The possibility of concentrating an analyte into a small volume of liquid is an advantage of LLE, because LSE without subsequent solvent evaporation does not usually allow concentration into a few microlitres of solvent. Thus, the sample preparation utilizes the extreme high mass sensitivity and overcomes the low concentration sensitivity of CE detection systems.

3.3. Validation results of CZE and HPLC methods

The bioanalytical CZE method was validated with respect to linearity of the detector response, method precision and accuracy, quantitation and detection limit, selectivity and recovery (see also Ref. [11]). Validation data for the CZE method are given in Table 1, example electropherograms in Fig. 5 and for the corresponding HPLC method in Table 2 and in Fig. 6.

The calibration equations and the calibration graphs for DTZ and M1 were linear over the

Table 1
Validation data for determination of diltiazem and desacetyldiltiazem in plasma with internal standard verapamil by CZE

Parameter	Desacetyldiltiazem				Diltiazem			
Nominal value (ng/ml)	5	10	50	250	5	10	50	250
Number of values	7	7	7	7	7	7	7	7
Average (ng/ml)	4.87	10.1	50.7	252.9	4.77	10.4	47.2	250.8
Accuracy (%)	−2.5	+1.4	+1.4	+1.2	−4.7	+3.7	−5.5	+0.3
Precision (%)	11.2	4.6	2.1	4.0	12.7	7.4	2.6	3.6
Correlation coefficient (*r*)	0.9994				0.9991			

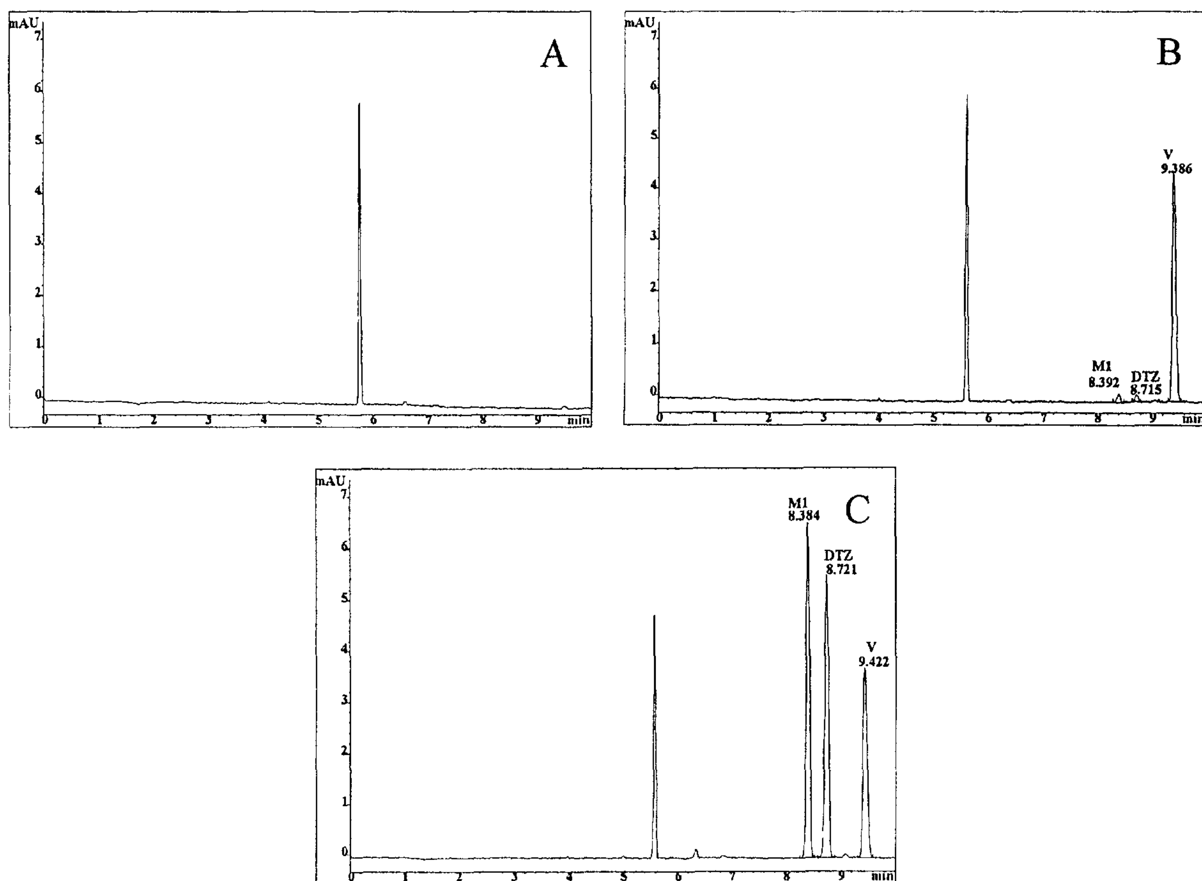

Fig. 5. Electropherograms of the extracts of three plasma samples. (A) Blank plasma without internal standard; (B) plasma sample containing 5 ng/ml desacetyldiltiazem (M1) and diltiazem (DTZ) plus internal standard verapamil (V); (C) plasma with 250 ng/ml M1 and DTZ, and with internal standard.

Table 2
Validation data for determination of diltiazem and desacetyldiltiazem in plasma with internal standard verapamil by HPLC

Parameter	Desacetyldiltiazem			Diltiazem		
Nominal value (ng/ml)	5	50	250	5	50	250
Number of values	10	7	7	10	7	7
Average (ng/ml)	5.2	50.5	242.8	5.1	50.4	243.9
Accuracy (%)	+4.0	+1.0	−2.9	+2.0	+0.8	−2.4
Precision (%)	12.5	2.2	3.8	10.4	3.4	4.0
Correlation coefficient (r)		0.9999			0.9999	

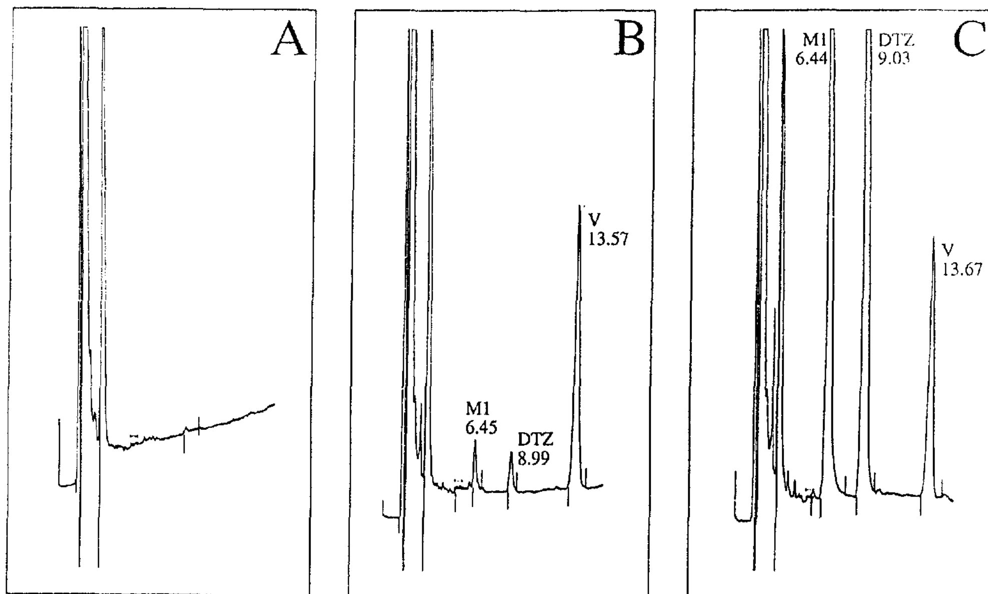

Fig. 6. Chromatograms of the extracts of three plasma samples. (A) Blank plasma without internal standard; (B) plasma sample containing 5 ng/ml desacetyldiltiazem (M1) and diltiazem (DTZ) plus internal standard verapamil (V); (C) plasma with 250 ng/ml M1 and DTZ, and with internal standard. Flow-rate, 1.0 ml/min; injection, 50 μl; column, Spherisorb ODS 2, 10 μm (250 × 4 mm I.D.); mobile phase, acetonitrile–0.01 *M* ammonium phosphate buffer (pH 3.7) (60:40); detection, 238 nm. Numbers on peaks are retention times (min).

range 5–250 ng/ml for both the HPLC and CZE methods with correlation coefficients greater than 0.999. The calibration equations were as follows:

M1 (CZE method):
$y = 5.4089 \cdot 10^{-3}x + 4.2581 \cdot 10^{-3}$
M1 (HPLC method):
$y = 2.19914 \cdot 10^{-2}x - 1.96434 \cdot 10^{-2}$
DTZ (CZE method):
$y = 5.0490 \cdot 10^{-3}x + 5.0335 \cdot 10^{-3}$
DTZ (HPLC method):
$y = 1.96033 \cdot 10^{-2}x + 3.813 \cdot 10^{-4}$

where y = area ratio value and x = concentration (ng/ml).

The detection limit (signal-to-noise ratio ≈ 3) was about 1.5 ng/ml for DTZ and M1 for the HPLC method and about 2 ng/ml for the CZE method. No interferences from plasma components were detected. Precision and accuracy were assessed by repeated sample preparation and analyses of plasma controls containing various concentrations of DTZ and metabolite. The precision and accuracy for plasma concentration below 10 ng/ml are less than 13% and for the range 10–250 ng/ml are less than 10% (Tables 1 and 2). The recovery for DTZ and M1 was about 70%.

3.4. Comparison

Although there is the advantage of better reproducibility for HPLC in comparison with CE using aqueous solutions, this has no impact on the precision and accuracy of the bioanalytical method. Also, the linearity, selectivity and long-term reliability of the two methods are the same. In terms of detection limit HPLC has a slight advantage over CZE, but this can be compensated by a further decrease in the back-extraction volume, which was checked down to 20 μl. An advantage for CZE is that there is a sufficient sample volume for several repeated analyses and less solvent consumption, and further only water-based buffers are used.

4. Conclusion

The HPCE assay described provides a selective and reliable method for the quantification of diltiazem and desacetyldiltiazem in plasma. By decreasing the back-extraction volume, the disadvantage of CE concerning the lower concentration sensitivity in comparison with HPLC could be eliminated. A quantitation limit of 5 ng/ml for diltiazem and desacetyldiltiazem in plasma could be achieved with good precision over the linear range 5–250 ng/ml. This method is a useful alternative to our already established HPLC method.

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