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Automatic determination of diltiazem and desacetyldiltiazem in human plasma using liquid–solid extraction on disposable cartridges coupled to HPLC — Part I: optimization of the HPLC system and method validation*

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Abstract: A sensitive and automated method for the analysis of diltiazem and desacetyldiltiazem in plasma has been developed using liquid–solid extraction (LSE) on disposable extraction cartridges (DECs) in combination with HPLC. After isolation from plasma, the analytes are separated on a highly deactivated octyl silica column with a mobile phase of methanol–0.05 M phosphate buffer (pH 7.4) (62:38, v/v). The analytes are monitored photometrically at 238 nm. The complete preparation of the plasma sample as well as the injection of the final extract on to the analytical column are performed automatically by means of a sample processor equipped with a robotic arm to which is attached a needle dispensing the different liquids. The internal standard solution is first added to the plasma sample. The DEC is then conditioned successively with methanol and phosphate buffer (pH 7.4). A 1.0-ml volume of sample containing the internal standard solution is applied on an extraction cartridge filled with cyanopropyl silica (50 mg). After the DEC has been washed with the same buffer, the analytes are eluted with 0.16 ml of methanol. A 0.14-ml volume of buffer is then passed through the DEC and 0.25 ml of the final extract is injected onto the HPLC column. The absolute recoveries of the drugs are about 90% and the limit of detection for diltiazem is 0.8 ng ml^{-1}. Relative standard deviations of 2.6% (within-day) and 3.7% (between-day) have been obtained for this compound at a plasma concentration of 50 ng ml^{-1}.

Keywords: *Diltiazem; desacetyldiltiazem; disposable extraction cartridges; deactivated stationary phase; automatic determination of drugs in plasma; liquid–solid extraction.*

Introduction

Diltiazem is a well-known calcium antagonist used in the treatment of angina pectoris, hypertension and supraventricular arrhytmias [1, 2]. Diltiazem and metabolites have been determined in plasma by conventional gas chromatography with NPD [3, 4] or ECD [5] detection, more recently by capillary gas chromatography with FID or ECD detection [6], and in most cases by HPLC with UV absorbance detection [6–23].

The methods described usually involve liquid–liquid extraction of plasma by organic solvents [3, 5, 9, 12, 21], after alkalinization [5, 6, 13, 17, 19, 20, 22, 23], as a sample clean-up procedure. In some cases, an aqueous acidic solution is used for back-extraction of diltiazem and its metabolites from the organic phase [8, 10, 11, 13, 15, 18–23]. A HPLC method using liquid–solid extraction (LSE) in a column-switching system has also been proposed [16].

The aim of the present paper is to describe an automatic method for the determination of diltiazem and desacetyldiltiazem (Fig. 1) in plasma, which combines LSE on disposable extraction cartridges (DECs) and HPLC. The influence of the composition of the HPLC

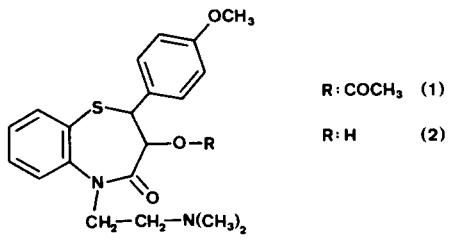

Figure 1
Structures of (1) diltiazem and (2) desacetyldiltiazem.

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system on analyte retention and peak shape as well as the stability of the analytical column have been studied. The method has also been validated.

Experimental

Apparatus

The HPLC system consisted of a Gilson model 305 solvent delivery system coupled to a Gilson model 116 UV detector (Villiers-le-Bel, France) capable of monitoring two wavelengths simultaneously.

The preparation of the plasma samples was performed by an ASPEC system (Automatic Sample Preparation with Extraction Cartridges) from Gilson.

The ASPEC system has three components: a set of racks (solvent rack, sample rack, LSE rack); a model 401 dilutor-pipettor; and an automatic sampling injector module which is equipped with an electrically actuated six-port Rheodyne valve and a robotic arm holding a needle through which the different liquids were aspirated and dispensed. The loop of the injection valve had a volume of 0.25 ml in the present method. The LSE rack consists of a DEC holder, a drain cuvette and a collection rack. The needle of the auto-sampler module can move the DEC holder in such a way that each DEC can be automatically placed above the drain cuvette during conditioning, sample loading and washing or above a collection tube during elution.

A LiChroCART analytical column (125 × 4 mm, i.d.) was used in combination with a short LiChroCART guard column (4 × 4 mm, i.d.), both prepacked with the same support material (Merck, Darmstadt, Germany). The guard column was connected to the analytical column by introducing it in the fittings of a Manu-CART system from Merck. The columns were thermostatted at 35.0 ± 0.1°C in a model 02PT923 water-bath from Heto (Birkeröd, Denmark).

An IBM compatible computer (PC-AT; CPU type: 80286), equipped with a 40 Mbyte hard disk, 1 Mbyte RAM, a Gilson software GME-714 version 1.3 (HPLC system controller), a Gilson software GME-718 version 1.1 (Sample manager) and an EPSON LX-800 printer, was used to control the HPLC and ASPEC systems as well as for data collection, storage and treatment.

A model BD9 two-channel recorder from Kipp and Zonen (Delft, The Netherlands) was also used for data collection.

Chemicals and reagents

Diltiazem, desacetyldiltiazem and propionyldesacetyldiltiazem (the internal standard) were supplied by the pharmaceutical company Galephar (Brussels, Belgium) and used without further purification.

Potassium monohydrogen phosphate and sodium hydroxide were of p.a. quality from Merck (Darmstadt, Germany). *N,N,N',N'*-Tetramethylethylenediamine was obtained from Riedel-de Haën (Seelze, Germany) and 1-octanesulphonic acid sodium salt from Sigma (St Louis, MO, USA). Methanol was of HPLC grade from Janssen (Geel, Belgium). Water was of Milli-Q quality (Millipore Corporation, Bedford, MA, USA).

Bond Elut DECs (capacity = 1 ml), prepacked with 50 mg of 40-μm cyanopropyl bonded silica, were used as supplied by Analytichem (Harbor City, CA, USA). When loaded with plasma, each DEC was used only once.

The LiChroCART guard and analytical columns were prepacked with 5-μm LiChrospher 60RP-Select B (C8) (Merck).

Chromatographic technique

The compounds of interest were separated in the isocratic mode, using methanol–phosphate buffer (pH 7.4) (62:38, v/v) as the mobile phase which was degassed for 10 min in an ultrasonic bath. The flow-rate was 1.0 ml min^{-1} and UV detection was carried out at 238 nm.

The phosphate buffer (pH 7.4) was prepared in a 1-l volumetric flask by mixing 250 ml of 0.1 M potassium dihydrogen phosphate with 195.5 ml of 0.1 M sodium hydroxide. The solution was diluted to 1 l with water. The pH of the buffer solution was measured before filtration through a nylon filter (0.45 μm) from Schleicher & Schuell (Dassel, Germany).

Standard solutions

Stock solutions of diltiazem, desacetyldiltiazem and propionyldesacetyldiltiazem (internal standard) were prepared in methanol at a concentration of 1 mg ml^{-1}. A mixed diltiazem and desacetyldiltiazem solution was made in water (concentration = 10 μg ml^{-1}) and further diluted with phosphate buffer (pH 7.4) (concentration: 0.2 or 1 μg ml^{-1}) to spike

plasma samples (calibration solutions). The stock solution of the internal standard was also diluted successively with water and the same buffer to the concentration of 1.2 μg ml^{-1}. Stock solutions were prepared once a month [11, 15, 16] whereas the water solutions were made up once a week and stored in a refrigerator at 4°C [16]. New internal standard and calibration solutions were prepared every day.

Automatic sample preparation

The only manual procedure was the centrifugation of the plasma sample at 6000 rpm for 10 min. A 2.0-ml volume of plasma was then introduced into a vial placed on the sample rack of the auto-sampler. The automatic procedure was then started.

Before the beginning of the first cycle, the needle of the auto-sampler and the external tubing of the injection valve were washed with 2.0 ml of phosphate buffer (pH 7.4). Between each step, the needle was rinsed with 1.0 ml of buffer (flow-rate: 24.0 ml min^{-1}) and a 10-mm air gap was generated inside the transfer tubing before pipetting the next liquid, in order to avoid cross-contamination.

The solvents and the plasma sample were dispensed under positive pressure and they were then pushed through the DEC by air. A special cap was placed on each DEC for ensuring air-tightness when liquid or air was dispensed through the needle. The dispensing flow-rates of liquids ranged from 0.18 to 96.0 ml min^{-1}. The air pressurizing volumes could also be varied, the passage of liquid through the DEC being accelerated by the use of larger air volumes.

Automatic sequence

The automatic sequence was performed in the following way (total cycle time = 18.4 min).

Addition of internal standard (flow-rate = 3.0 ml min^{-1}; bubbling air volume = 1.0 ml). A 0.05 ml volume of internal standard solution (concentration: 1.2 μg ml^{-1}) was aspirated from the solvent rack and dispensed in the plasma sample which was then homogenized by bubbling.

DEC conditioning (flow-rate = 6.0 ml min^{-1}; air volume = 0.3 ml). At the start, the DEC holder was located above the drain cuvette (front position). The DEC (Bond Elut CN, 50 mg) was treated successively with 1.0 ml of methanol and 1.0 ml of phosphate buffer (pH 7.4).

Loading with plasma sample (flow-rate = 0.18 ml min^{-1}; air volume = 0.3 ml). A 1.0 ml volume of plasma was aspirated from the corresponding vial and dispensed on the DEC.

Washing (flow rate = 1.5 ml min^{-1}; air volume = 0.6 ml). 1.0 ml of phosphate buffer (pH 7.4) was applied on the DEC.

Elution (flow rate = 1.5 ml min^{-1}; air volume = 0.6 ml). The DEC holder was pushed by the needle over the collection rack. A 0.16 ml volume of methanol was dispensed on the DEC. The eluate was collected in the tube positioned under the DEC.

Addition of buffer (flow rate = 1.5 ml min^{-1}; air volume = 0.6 ml). A 0.14 ml volume of phosphate buffer (pH 7.4) was passed through the DEC. Afterwards the DEC holder was replaced in its front position.

Mixing. The resulting elute was successively aspirated and dispensed in the collection tube. These operations were repeated two times.

Injection. The whole volume of the final extract was aspirated from the collection tube and dispensed in the loop filler port. By switching of the injection valve, 0.25 ml of the extract was injected on to the HPLC column, the excess being directed to the waste.

Chromatographic separation. The chromatographic separation of the prepared sample was performed during the preparation of the next sample (concurrent mode).

Results and Discussion

Optimization of the HPLC system

Diltiazem and its main metabolite, desacetyldiltiazem, are basic compounds with a tertiary amino-group (Fig. 1). As could be expected with this kind of compound, broad and asymmetrical peaks have been obtained on alkyl bonded silica (Fig. 2, A), owing to interactions of these compounds with residual silanol groups at the silica surface. These detrimental effects can be reduced considerably either by addition to the mobile phase of a

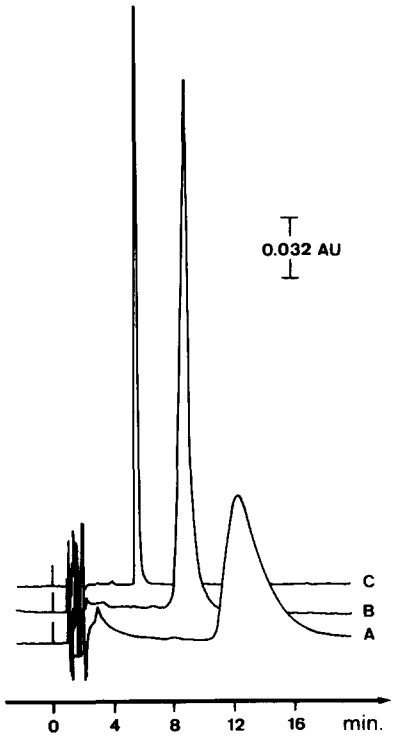

Figure 2
(A) Solid phase: Lichrospher RP 18; mobile phase: phosphate buffer (pH 3.0)–methanol (60:40, v/v). (B) Solid phase: Lichrospher RP 18; mobile phase: 0.05 M TEMED in phosphate buffer (pH 4.2)–methanol (50:50, v/v). (C) Solid phase: Lichrospher RP-Select B; mobile phase: phosphate buffer (pH 3.0)–methanol (50:50, v/v).

competing aliphatic amine [24] or by using a highly deactivated bonded silica as stationary phase. As can be seen in Fig. 2, the addition of tetramethylethylenediamine (TEMED) (B) to the mobile phase gives rise to an improvement in the symmetry of the diltiazem peak but (C) shows that even better results with respect to peak symmetry and efficiency can be obtained by the use of a deactivated stationary phase (Lichrospher RP-Select B).

As shown in Table 1, the retention of diltiazem, expressed by its capacity ratio, k', increases with the pH of the phosphate buffer included in the mobile phase, owing to reduction of the ionization of the amino-group. An increase in retention can also be obtained by adding an ion-pairing agent, such as the anion octanesulphonate, to the mobile phase buffer (Table 1). A low pH (3.0) is used in this case so that diltiazem is essentially present in the mobile phase in the ionized form. The same retention behaviour has been observed for desacetyldiltiazem but the latter compound

Table 1
Influence of the mobile phase composition on the capacity ratio (k') of diltiazem

Buffer pH	O.S. conc.	k' at different methanol concentrations (%) 50	55	60	62	65
3.0	—	4.8	—	—	—	—
3.0	5×10^{-3} M	7.7	4.6	2.6	2.1	—
3.0	2.5×10^{-2} M	—	5.9	4.1	3.5	2.8
6.0	—	—	—	8.3	7.6	—
7.4	—	—	—	11.8	9.6	6.5

Solid phase: 5-μm Lichrospher 60 RP-Select B. Mobile phase: phosphate buffer (containing sodium octanesulphonate: O.S.)–methanol.

gives significantly lower capacity ratios than diltiazem (Fig. 3).

Retention data in Table 1 clearly show, however, that the change of pH from 3.0 to 7.4 gives rise to a more pronounced increase in k' than does the addition of octanesulphonate. Such an effect is favourable in the present method as it permits the use of a higher concentration of methanol in the HPLC mobile phase. Under these conditions, the volume of buffer which must be added to the methanolic eluate in the last step of the preparation procedure can be minimized. This buffer addition is needed to decrease the eluting strength of the methanolic extract before its injection into the HPLC system [25]. Obviously, high methanol concentrations in the mobile phase will reduce the volume of buffer to be added and limit the dilution of the plasma extract.

Stability of the HPLC column

As observed previously in similar systems [26, 27], column efficiency and consequently analyte peak height have a tendency to decrease with increasing total volume of plasma extract introduced in the chromatographic system. This loss of efficiency is accompanied by an increase of column back pressure.

The lifetime of the analytical column can be extended by replacing regularly the short guard column, which is packed with the same packing material [27]. In the present method, the guard column could be loaded with more than 20 ml of plasma extract (i.e. 80 injections) without significant deterioration of the analytical column. However, in order to maximize the lifetime of the HPLC column, it is advisable to change the guard column after 60 injections. Under these conditions, the replacement of the

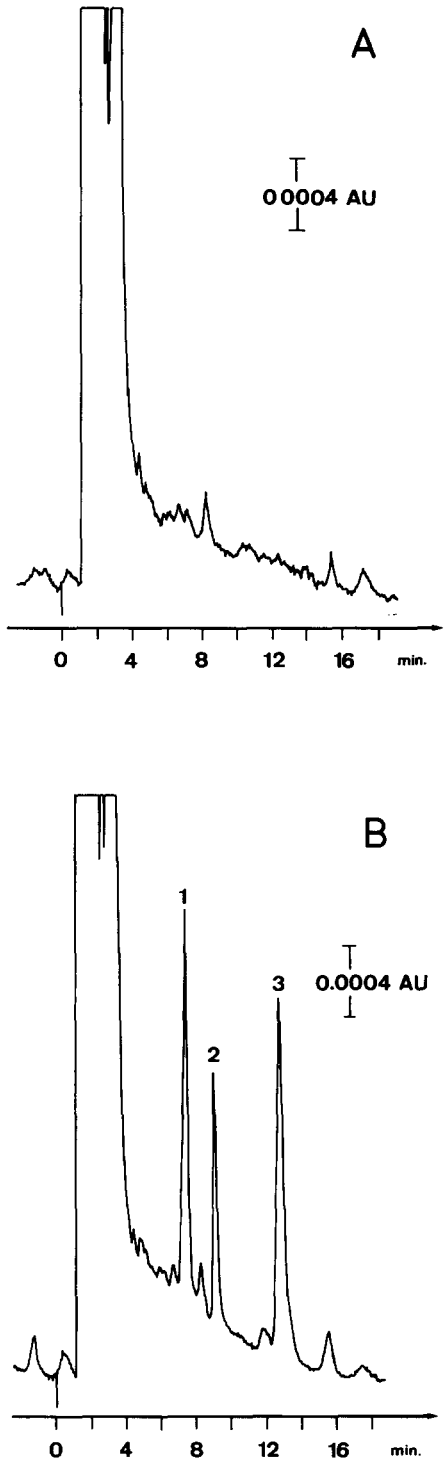

Figure 3
Typical chromatograms obtained by using LSE on disposable extraction cartridges coupled to HPLC. Mobile phase: phosphate buffer (pH 7.4)–methanol (38:62, v/v); solid phase: 5-μm LiChrospher 60 RP-Select B; UV detection: 238 nm; DEC: Bond Elut CN 50 mg; total extract volume: 0.30 ml. Samples: (A) blank plasma; (B) spiked plasma. Peaks: (1) desacetyldiltiazem: 8.3 ng (10 ng ml^{-1}); (2) diltiazem: 8.3 ng (10 ng ml^{-1}); (3) propionyldesacetyldiltiazem (IS): 50.0 ng (60 ng ml^{-1}).

analytical column was necessary only after the injection of a total volume of plasma extract of about 90 ml, which corresponds approximately to 360 assays.

Detection

In all previously published HPLC methods [6–23], the determination of diltiazem and its metabolites in plasma was performed by UV detection, the wavelength of measurement ranging from 210 [5] to 254 nm [10]. Under the chromatographic conditions described above, an absorbance maximum was observed at 238 nm for diltiazem as well as for desacetyldiltiazem and the internal standard propionyldesacetyldiltiazem. Consequently, this wavelength was selected in the present method.

Validation of the method

Under the conditions described above, desacetyldiltiazem, diltiazem and propionyldesacetyldiltiazem have mean capacity ratios (k') of 7.7, 9.5 and 13.9, respectively.

Absolute recovery. As can be seen in Table 2, the absolute recoveries of the two analytes, determined at the 100 ng ml^{-1} concentration level, are about 90%. They were estimated by comparing the peak heights obtained after extraction of spiked plasma samples by the method described, with those found on direct injection of aqueous standard solutions by use of the same autosampler.

Linearity. Linear regression analysis for diltiazem and desacetyldiltiazem made by plotting the peak height (Y) versus the concentration (X) in ng ml^{-1} gives the following equations (238 nm; AUFS = 0.005; concentration range = 7.5–100 ng ml^{-1}; n = 7):

$$\text{diltiazem: } Y = 26.4X + 3.2, \quad r^2 = 0.99912$$
$$\text{desacetyldiltiazem: } Y = 30.7X - 19.6, \quad r^2 = 0.99783.$$

Table 2
Absolute recovery of desacetyldiltiazem and diltiazem*

	Recovery (%)	
	Desacetyldiltiazem	Diltiazem
Mean	88.7	91.9
SD	2.7	2.5

*Concentration of each compound = 100 ng ml^{-1}; n = 10.

The relationship between peak height and concentration is linear at least up to 500 ng ml^{-1}.

Sensitivity. Limits of detection (LOD) and of quantitation (LOQ) [28] were calculated from regression lines obtained in the low concentration range (up to 50 ng ml^{-1}). The LOD for diltiazem is equal to 0.8 ng ml^{-1} and the LOQ to 2.6 ng ml^{-1}. The LOD and LOQ for desacetyldiltiazem are slightly higher, 2.1 ng ml^{-1} and 7.2 ng ml^{-1}, respectively.

Reproducibility. The precision of the method was estimated by repeated analysis of spiked plasma samples containing different concentrations of diltiazem and desacetyldiltiazem. The results obtained for within-day and between-day reproducibilities are presented in Table 3. In most cases, desacetyldiltiazem gives rise to slightly higher relative standard deviations than does diltiazem.

Table 3
Within-day and between-day reproducibilities

Analyte concentration (ng ml^{-1})	n	Relative standard deviation (%) Desacetyldiltiazem	Diltiazem
Within-day reproducibility			
100	6	2.2	2.6
50	5	1.5	2.6
25	5	5.1	3.3
10	5	5.2	3.0
5	5	3.9	3.4
Between-day reproducibility			
100	9	2.4	2.2
50	9	4.0	3.7
25	9	7.9	3.6
10	9	6.3	4.1
5	9	8.1	5.6

Selectivity. At the retention time of the two analytes, the absence of interfering endogenous components is demonstrated in Fig. 3 which shows typical chromatograms obtained on injection of blank and spiked plasma samples (10 ng ml^{-1}).

Application of the automated method

The present automated method has been used to determine the concentration of diltiazem and its desacetyl metabolite in plasma samples within the framework of pharmacokinetic studies in humans after oral administration. It has been applied to more than 1500 determinations and has proved to be rugged.

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