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

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Abstract: An automatic liquid–solid extraction (LSE) procedure to be coupled to HPLC for the determination of diltiazem and desacetyldiltiazem in plasma has been developed. The LSE operations are performed on disposable extraction cartridges (DECs) by means of a sample processor equipped with a robotic arm holding a needle through which the different liquids are dispensed. The operating parameters of LSE have been optimized with respect to recovery, detectability and reproducibility by using, whenever possible, aqueous solutions of the analytes. Different kinds of DECs have been tested. For the compounds studied, DECs filled with 50 mg of cyanopropyl silica have been selected. The influence of the pH of the buffer used in the washing step has been studied, leading finally to the selection of the same phosphate buffer (pH 7.4) as in the HPLC mobile phase. The minimum volume of methanol which still gives a nearly complete elution of the analytes from the extraction cartridges has been determined. Under these conditions, a high sensitivity can be obtained without an evaporation step. Moreover, the volume of buffer to be added to the methanolic eluate before injection into the HPLC system has been optimized in such a way that a focusing effect is obtained at the top of the analytical column while the dilution of the extract is minimized.

Keywords: *Diltiazem; desacetyldiltiazem; disposable extraction cartridges; automatic determination of drugs in plasma; automated liquid–solid extraction coupled to HPLC; aqueous standard solutions for method optimization.*

Introduction

When traces of drugs must be determined in complex matrices, such as biological fluids, a sample handling procedure is usually needed before the HPLC separation. The main objectives of this sample pretreatment are the removal of proteins, which can cause deterioration of the chromatographic column, the elimination of interfering matrix components and the concentration of the sample.

Different methods of sample preparation such as deproteinization, ultrafiltration, addition of a proteolytic enzyme, dilution, liquid–liquid extraction (LLE) and liquid–solid extraction (LSE) or solid-phase extraction (SPE) can be used [1–7]. When they are performed manually, these sample preparation techniques are often tedious and time-consuming. Therefore, techniques with high automation potential, such as LSE, are of particular interest when the number of samples is relatively large. In addition, the development of an automated sample handling procedure often leads to better results with respect to accuracy and precision. Automated LSE techniques for sample preparation are based on the use of either a column-switching system [1, 3, 7–9] or disposable extraction cartridges (DECs) [10–12].

The ASPEC system (Automatic Sample Preparation with Extraction Cartridges) permits full automation of the determination of drugs and related compounds in plasma or serum by on-line coupling of LSE on DECs to HPLC [2, 5, 6, 7, 11, 12]. All LSE operations as well as the addition of an internal standard and the injection of the final extract on to the HPLC column are performed automatically by the sample processor [11, 12].

After selection of suitable chromatographic and detection conditions [13], most steps of the

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automatic LSE procedure can be optimized by the use of aqueous standard solutions of the analytes in place of spiked plasma samples [14]. This limits considerably the consumption of DECs since the latter can be reused many times with aqueous solutions whereas they can generally be used only once after loading with plasma.

This paper deals with the optimization of the different LSE steps, using DECs and aqueous standard solutions, with the aim of developing a fully automated method for the determination of diltiazem and desacetyldiltiazem in plasma.

Experimental

Apparatus

The chromatographic equipment comprised a Gilson model 305 pump (Villiers-le-Bel, France), a LiChroCART analytical column (125 × 4 mm, i.d.) preceded by a short LiChroCART guard column (4 × 4 mm, i.d.) from Merck (Darmstadt, Germany), a model 02PT923 water-bath from Heto (Birkerød, Denmark) and a Gilson model 116 UV detector. This was used in combination with an ASPEC system (Automatic Sample Preparation with Extraction Cartridges) from Gilson [11, 12].

An IBM compatible computer which was equipped with GME-714 and GME-718 softwares (Gilson) and a model BD9 recorder (Kipp and Zonen, Delft, The Netherlands) were also used.

Chemicals and reagents

All reagents were of analytical grade from Merck (Darmstadt, Germany).

Diltiazem, desacetyldiltiazem and propionyldesacetyldiltiazem (the internal standard) were supplied by the pharmaceutical company Galephar (Brussels, Belgium) and used without further purification. 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) filled with 50 or 100 mg of 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 both prepacked with 5-μm LiChrospher 60RP-Select B (C8) (Merck).

Chromatographic technique

The mobile phase consisted of methanol–phosphate buffer (pH 7.4) (62:38, v/v). UV detection was performed at 238 nm. Further experimental details are given in Part I [13].

Standard solutions

Stock solutions of diltiazem, desacetyldiltiazem and propionyldesacetyldiltiazem (internal standard) prepared in methanol at a concentration of 1 mg ml^{-1} were made up once a month [15]. A mixed diltiazem and desacetyldiltiazem solution was made in water (concentration = 10 μg ml^{-1}) weekly. It was further diluted with phosphate buffer (pH 7.4) to a concentration of 100 ng ml^{-1}.

A new standard solution in buffer was prepared every day. The preparation of calibration and internal standard solutions for spiking plasma samples is described in Part I [13].

Automatic sample preparation

The experimental conditions for the automated sample preparation procedure are detailed in Part I [13]. A summary of these conditions is given in Table 1.

Table 1
Automatic sample preparation procedure*

Step	Liquid	Volume (ml)	Dispensing flow rate (ml min^{-1})
Internal standard addition	Internal standard solution	0.05	3.00
Conditioning	Methanol	1.00	6.00
	Buffer (pH 7.4)	1.00	6.00
Sample loading	Plasma	1.00	0.18
Washing	Buffer (pH 7.4)	1.00	1.50
Elution	CH_3OH	0.16	1.50
Buffer addition and mixing	Buffer (pH 7.4)	0.14	1.50
Injection loop loading	Plasma extract	0.30	0.75

*DEC: Bond Elut CN (50 mg).

Table 2
Type and mass of sorbent used in the disposable extraction cartridges*

Type of sorbent	Mass of sorbent (mg)	Recovery of diltiazem (%)
$C18^{EC}$	100	93.0
C18	100	93.0
C8	100	92.4
C2	100	98.7
CN	100	96.6
C18	50	95.7
CN^{EC}	50	99.1
CN	50	95.4

*Conditions — DECs: Bond Elut (50 or 100 mg); conditioning: methanol–buffer pH 7.4 (1.0 ml of each); washing: buffer pH 7.4; elution: 0.62 ml of methanol; buffer addition: 0.38 ml of buffer (pH 7.4); sample: aqueous standard solution of diltiazem (100 ng ml^{-1}); other conditions as given in Experimental.

The minimum dispensing flow rate available (0.18 ml min^{-1}) has been automatically selected for the sample loading step [12].

Results and Discussion

Selection of the LSE sorbent

As can be seen in Table 2, various kinds of DECs containing bonded silicas with widely different polarities have been tested. Aqueous solutions of diltiazem have been used as samples and the corresponding recoveries of the drug have been measured. At this stage, the different steps of the LSE procedure had not yet been optimized. A phosphate buffer (pH 7.4), previously selected in a similar automated method for the bioanalysis of indomethacin [12], was used in the conditioning, washing and buffer addition steps. The elution step was performed with 0.62 ml of methanol and subsequently 0.38 ml of buffer was passed through the cartridge in order to obtain a final extract with the same eluting strength as the HPLC mobile phase [13]. Under these preliminary conditions, recoveries higher than 90% have been obtained for diltiazem with each sorbent tested (Table 2).

Table 2 also shows that DECs packed with 50 mg of sorbent give rise to similar recoveries as those filled with 100 mg. Like the more commonly used 100 mg DECs, the 50-mg extraction cartridges can be loaded with 1.0 ml of plasma sample. However, the latter require smaller volumes for their elution; this results in an increased sensitivity since no evaporation step is used. Therefore, DECs containing 50 mg of sorbent are preferred.

In order to select the most appropriate sorbent with respect to selectivity, spiked plasma samples were applied on each of the 50 mg DECs investigated (Table 2) under the same preliminary conditions. The hydrophobic octadecyl silica phase gave rise to chromatograms with several sources of interference in the vicinity of the analyte peaks. On the contrary, very clean chromatograms devoid of interfering peaks from plasma components have been obtained using DECs packed with ordinary cyanopropyl silica (CN). Poorer selectivity has been found with the endcapped cyanopropyl bonded phase (CN^{EC}). In spite of the particularly high recoveries obtained with the latter sorbent (Table 2), DECs filled with ordinary cyano phase were chosen.

Washing step

Table 3 shows the influence of the pH of the buffer used as washing eluent on the recovery of diltiazem and desacetyldiltiazem. In all cases, the conditioning of the DEC before

Table 3
Selection of buffer pH for the washing step

Buffer pH	Recovery (%) Desacetyldiltiazem	Diltiazem
9.0	102.3	93.1
7.4	97.3	96.5
6.0	97.6	96.4
3.0	40.8	75.6

*Conditions — DEC: Bond Elut CN (50 mg); conditioning: use of the same buffer as for the washing step; elution: 0.62 ml of methanol; buffer addition: 0.38 ml of buffer (pH 7.4); sample: aqueous standard solution of diltiazem and desacetyldiltiazem (100 ng ml^{-1}); other conditions: as given in Experimental.

sample loading was performed with the same buffer as for the washing step.

With DECs filled with the fairly polar cyano phase, a significant decrease in the drug recoveries was observed by using a washing eluent of pH 3 (Table 3). However, such a decrease did not occur with DECs packed with a C_{18} phase. No significant changes in analyte recoveries were obtained with washing eluents of pH ranging from 6 to 9. The phosphate buffer (pH 7.4) was preferred as it is also present in the HPLC mobile phase [13]. Indeed, differences in pH between the washing eluent and the buffer used in the HPLC mobile phase can sometimes cause the appearance of disturbing system peaks on the chromatograms [9].

With 50-mg cartridges packed with cyanopropyl silica, the volume of buffer (pH 7.4) used in the washing step had to be limited to 1.0 ml since a significant decrease in the analyte recoveries was observed when a 2.0 ml volume was used. Again, such a change in the volume of washing eluent had no effect on the recoveries in the case of DECs filled with octadecyl silica.

Similarly, the selectivity of the washing step could not be further enhanced by addition of methanol to the washing eluent when the DECs containing the cyano phase were used in the LSE procedure. The recoveries of diltiazem and of desacetyldiltiazem were found to decrease at methanol concentrations higher than 10% (v/v).

Elution step

Pure methanol was selected for the elution step, as mixtures of methanol with more than 20% (v/v) of phosphate buffer (pH 7.4) had too low eluting strengths to give analyte recoveries higher than 90%.

In order to improve the detectability of diltiazem, the minimum volume of methanol which still gives a practically complete elution of the compound from the DEC had to be determined.

As can be seen from Fig. 1, 0.16 ml of methanol still gave rise to a maximum recovery of diltiazem and was thus selected. After buffer addition to obtain the same eluting strength as in the HPLC mobile phase (methanol–buffer, pH 7.4, 62:38, v/v), the analyte concentration in the final extract was then 3.9 times higher than in the sample. It should be emphasized that when such small volumes of methanol were used for the elution of the analytes, a constant volume of eluate, equivalent to the volume of methanol dispensed, could only be obtained if the buffer to be added was also passed through the DEC [14]. When only methanol was applied onto the DEC and buffer was added directly to the collection tube, the volume of eluate was often smaller, giving rise to anomalously high (more than 100%) and poorly reproducible recoveries.

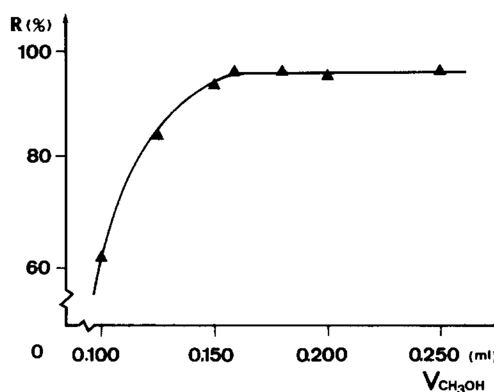

Figure 1
Minimum volume of methanol for the elution step. DEC: Bond Elut CN (50 mg); other conditions as given in Experimental; R: absolute recovery of diltiazem; VCH_3OH: volume of methanol.

Concentration effect on the HPLC column

Figure 2 shows that the addition of increasing volumes of buffer (pH 7.4) to the methanolic eluate (0.16 ml) before injection onto the HPLC column causes an increase in peak height because of a focusing effect at the top of the column.

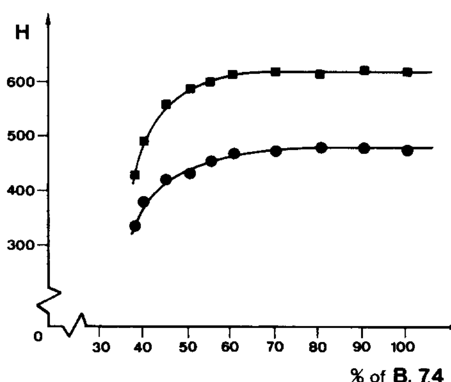

Figure 2
Increase of the peak height due to a concentration effect at the top of the column. Volume of methanol for the elution: 0.16 ml; other conditions as given in Experimental; H: corrected peak height (arbitrary units); % of B. 7.4: per cent of buffer (pH 7.4) in the final extract; (●): diltiazem; (■): desacetydiltiazem.

With larger buffer volumes than those corresponding to 55% of the total extract, no further increase in peak height was observed (Fig. 2). On the other hand, buffer addition increases the total volume of the extract, giving rise to a dilution and, consequently, to a decrease in sensitivity.

By addition of a buffer volume of 0.14 ml, corresponding to 47% of the total extract, the dilution was minimized (concentration factor = 3.3 instead of 3.9) and the peak height was 1.7 times higher than that obtained by injection of an extract with the same eluting strength as the HPLC mobile phase.

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