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Bioequivalence assessment of diltiazem preparations by means of discriminant analysis of data from solid-phase extraction and liquid chromatography*

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Abstract: A solid-phase extraction technique for sample clean-up coupled with a new LC procedure is reported for the assay of diltiazem in plasma. The use of disposable cartridges provides selective extraction and easy automation. A new LC system based on LiChrospher® RP 60 Select B columns is described. For routine analysis, the procedure provides a rapid simultaneous clean-up of several samples prior to chromatography and reproducible recoveries over a concentration range of 10–800 ng. The procedure was used to analyse the plasma samples from a bioequivalence study of three commercial diltiazem preparations. The pharmacokinetic parameters in 12 healthy male volunteers were determined and the assessment of bioequivalence was conducted by discriminant analysis.

Keywords: *Diltiazem preparations; bioequivalence; solid-phase extraction; liquid chromatography; discriminant analysis.*

Introduction

Diltiazem is a calcium antagonist, used in the treatment of variant angina [1, 2], with anti-arrythmic [3] and antihypertensive effects [4]. In the last 13 years a number of methods for the assay of diltiazem concentrations in human plasma have been published. Gas chromatographic methods using packed columns with NPD [5, 6] and ECD detection [7, 8] or capillary columns [9], although very sensitive, require prechromatographic derivatization. Thin layer chromatography has been used in some instances [10, 11] and, recently, chiral separation of diltiazem hydrochloride enantiomers was achieved by capillary zone electrophoresis [12]. In spite of the high efficiency of these methods, liquid chromatography is the analytical method most often used in the determination of plasma levels of diltiazem.

Several LC methods have been reported using normal phase [13, 14], reversed phase [15–24] with and without modifiers [24, 26], and for the resolution of optical isomers in quality control [25]. The procedure of Verghese *et al.* [27] has become quite popular, but the cyanoalkyl columns tend to produce severe peak tailing and become unusable after some time. All these methods are associated with laborious sample clean-up procedures that involve organic solvent extraction and back extraction, requiring 1–2 h sample preparation time, long equilibration times for some eluents [26], and in some instances, derivatization [13, 25]. An automated LC method for direct injection of plasma samples with column switching and on-line sample clean-up has been reported [28, 29], but requires some operational sophistication.

Solid-phase extraction represents a valuable alternative to the time consuming liquid–liquid extraction of drugs from the sample matrix [30]. It is quick, simple to operate, and allows simultaneous work-up of many samples. Most sample losses are avoided and automation is possible. Recently, solid-phase extraction was used in the determination of plasma levels of diltiazem and its metabolites and the results were compared with those obtained by the classical liquid–liquid extraction procedure [31]. A new method for sample clean-up based on solid-phase extraction of diltiazem from

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plasma samples coupled with reversed-phase LC analysis with LiChrospher® RP 60 Select B columns is described here. This type of column requires no mobile phase modifier and has good longevity. The method allows simultaneous clean-up treatment of many samples in few minutes followed by 8-min LC runs. It is applicable to bioavailability and bioequivalence studies. The bioequivalence of three commercial diltiazem preparations was assessed by a multivariate analysis of the plasma drug concentrations determined using the modified assay described.

Experimental

Materials

Diltiazem, *d*-3-acetoxy-*cis*-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(*p*-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one, hydrochloride and verapamil, α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-3,4-dimethoxy-(1-methylethyl)-benzeneacetonitrile, hydrochloride (internal standard) were provided by Instituto Luso-Farmaco (Lisbon, Portugal). All solvents were HPLC grade from Koch-Light Ltd (Haverhill, Suffolk, UK). All other reagents were analytical grade from E. Merck (Darmstadt, Germany). For the solid-phase extraction, Adsorbex® RP-18 (100 mg) disposable cartridges from E. Merck (Darmstadt, Germany) were used. A Model Adsorbex® SPU vacuum manifold from E. Merck (Darmstadt, GFR) was used for the simultaneous clean-up of 24 samples. Plasma samples were prepared from blood samples collected from volunteers and immediately frozen at −30°C until use. Three different commercial brands of tablets containing 60 mg of diltiazem hydrochloride were used in the bioequivalence assays. Standard solutions of diltiazem hydrochloride (0.1 mg 100 ml^{-1}) and verapamil hydrochloride (1.5 mg 100 ml^{-1}) in methanol were prepared.

Apparatus

The LC equipment consisted of a Gilson Model 302 5SC pump, a Gilson Model 802 manometric module, and a Model 7125 Rheodyne injection valve with a fixed volume loop of 50 μl. The effluent was monitored at 237 nm by means of a Shimadzu Model SP-2 variable wavelength detector, operated at 0.005 a.u. Quantitative measurements were made with a Shimadzu Model CR3-A computing integrator, equipped with a disk drive and a CRT monitor.

Sample preparation

Calibration. The appropriate amount of a standard methanolic solution of diltiazem hydrochloride (10, 25, 50, 100, 250, 500 and 800 μl) was transferred to 1-ml vials and the solvent was evaporated under a light stream of nitrogen. Blank plasma (1 ml) was added to the residue. The solution was applied to an Adsorbex® RP-18 cartridge containing 100 mg of C_{18} sorbent that had been previously conditioned with 1 ml of methanol followed by 1 ml of carbonate buffer (pH 10.5), and was drawn through the cartridge under vacuum to an Adsorbex® SPU assembly fitted with 3-ml reservoirs. The column was washed with 50 μl of carbonate buffer (pH 10.5), 100 μl of water, and 50 μl of methanol and the effluents were discarded. The retained diltiazem hydrochloride was eluted from the column with 2 ml of acetonitrile under a vacuum of 13 mm Hg, applied until the column appeared to be dry. The acetonitrile solution was collected, the solvent was evaporated under a light stream of nitrogen, and 50 μl of standard verapamil hydrochloride solution was added to the residue. A 50 μl aliquot of this solution was directly injected for chromatographic analysis. This procedure was used to evaluate the accuracy of the calibration curve on a day-to-day basis.

Preparation of plasma samples. Thawed plasma samples (1 ml) were applied to conditioned columns. The procedure for sample clean-up was identical to that described for calibration.

Chromatography

The chromatographic assays were performed with a LiChrospher® 60 RP Select B column (5 μm, 250 × 4.6 mm i.d., E. Merck). The mobile phase was a mixture of 312 g of acetonitrile and 680 g of phosphate buffer, pH 2.3. The buffer was made by dissolving 6.66 g KH_2PO_4 and 4.8 g of 85% H_3PO_4 in 1000 ml of water. The flow was 2 ml min^{-1}. A LiChrosorb® RP-18, 5 μm 4 × 4 mm column (E. Merck) was used as the guard column.

Discriminant analysis

Multivariate methods of pattern recognition were applied to the group classification of the

diltiazem preparations, composed of an assembly of features. Maximal plasma concentration in ng ml^{-1} (C_{max}), time after intake at which maximal concentration was reached (T_{max}), half-life time ($T_{½}$), area under the curve from 0–12 h after intake (AUC_{0-12}), and area under the curve extrapolated to infinity ($AUC_{0-\infty}$) were entered for each experiment as the corresponding mean values for each preparation. Calculations were performed by means of statistical software packages: Statgraphics (Statistical Graphics Corp., Rockville, MD) and Gbstat (Dynamic Microsystems Inc., Silver Spring, MD).

Bioavailability assay

Twelve healthy male volunteers, 20–33 years old, participated in a randomized crossover experiment in three phases. All of the participants were previously briefed on the experimental conditions and objectives and gave their consent in writing. During the phases of the study, each volunteer received a single 60 mg oral dose of a commercial diltiazem preparation. Three different preparations were studied, labelled as A, B and C, and were administered at a 1 week interval. During the time of the study, all other medication was absent and the ingestion of alcoholic beverages in the 24 h before administration was excluded. The volunteers were placed on a controlled diet and fasted 8 h before drug administration.

Blood samples were taken immediately before drug administration and at 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12 and 24 h after intake. The blood samples were collected in heparinized tubes, centrifuged immediately and stored at −30°C until analysis. The concentration value for each sample was taken as the mean of three replicates.

Results and Discussion

Specific and sensitive methods of analysis of samples from biological origin are strongly dependent on sample preparation. In the analysis of drugs in biological fluids, the drug is traditionally removed from the matrix by tedious liquid–liquid extraction procedures. In such methods, sample losses due to incomplete phase transfer are unavoidable. An alternative to the liquid–liquid extraction is solid-phase extraction. Although 100% recovery is not always possible, the method is rapid, easy to operate, reproducible, and can be easily automated. Moreover, by the use of appropriate manifolds, many samples can be simultaneously worked-up and no expensive equipment is required [30].

Figure 1 shows a typical chromatogram of a standard solution eluted under isocratic conditions. The peaks for diltiazem and the internal standard were well-separated and the chromatographic run was completed in about 8 min. This is especially important for bioavailability studies, where a high number of samples must be analysed in a short time. The peaks were well-shaped and the column maintained its performance after 6 months of regular use. This contrasts with the performance of cyano columns [27] which, after some time, tend to produce severe peak tailing with reduced performance even in the presence of the triethylamine modifier [20].

Figure 2 shows typical chromatograms of (A) plasma spiked with diltiazem hydrochloride and the internal standard verapamil hydro-

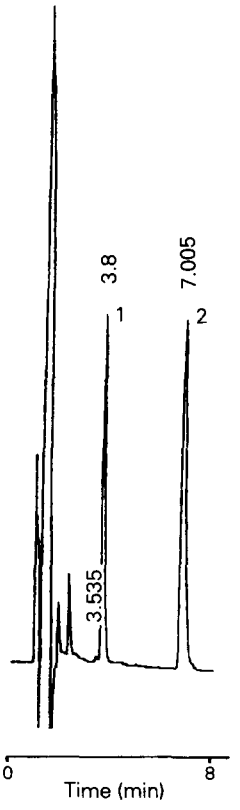

Figure 1
Chromatogram of a standard solution of 250 ng diltiazem hydrochloride (Peak 1) containing 750 ng of verapamil hydrochloride (Peak 2) as the internal standard. See text for chromatographic conditions.

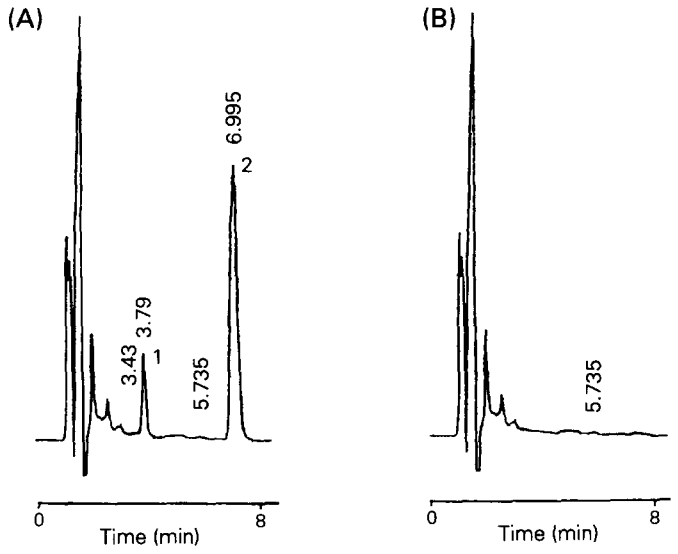

Figure 2
(A) Chromatogram of plasma (50 μl) spiked with (1) 100 ng of diltiazem HCl and (2) 750 ng of verapamil HCl. (B) Chromatogram of human plasma. See text for chromatographic conditions.

chloride and (B) human plasma. Plasma samples were free of endogenous interfering substances at the retention times corresponding to the analysed compounds.

The linearity of the detector response was tested for a diltiazem concentration range of 10–800 ng (Table 1). Standard solutions containing 10, 25, 50, 100, 250, 500 and 800 ng of diltiazem hydrochloride and a fixed amount (750 ng) of verapamil hydrochloride in 1 ml methanol were prepared. The peak area ratios obtained after injection of 50 μl aliquots were calculated. One straight line was obtained. The regression equation was $y = ax + b$ where y is the peak area ratio of diltiazem to internal standard and x is the weight ratio of spiked diltiazem hydrochloride to the internal standard weight. The calculated equation was $y = 1.6950\,x - 0.0089$ ($r = 0.9955$, SEM 0.3081, $n = 6$). A slight deviation was observed for concentrations of diltiazem above 750 ng, although the values were within the 95% confidence level. Such high values are not likely to occur in plasma samples within the normal therapeutic levels [32].

For the analysis of plasma samples, a calibration curve was constructed as follows: 10, 25, 50, 100, 250, 500 and 800 ng of diltiazem hydrochloride were dissolved in 1 ml of human plasma. The solutions were loaded onto the solid-phase extraction cartridges as described in the Experimental section. A linear detector response was obtained for the concentration range 10–800 ng ml^{-1}. The linear regression line corresponded to equation $y = 1.369\,x - 0.019$ ($r = 0.9990$, SEM = 0.2065, $n = 7$) with a mean recovery of 81% (RSD = 2.1%). The detection limit at S/N = 4 was 0.5 ng at maximal detector sensitivity.

The internal standard was added to the dry extract in order to calculate absolute rather than relative recoveries. This was important

Table 1
Linearity of detector response for standard solutions of diltiazem hydrochloride in a concentration range of 10–800 ng ml^{-1} ($n = 6$)

Theoretical concentration	Found concentration	RSD (%)	RE (%)
10	10.8	10	6
25	24.8	11	7
50	51.1	4	4
100	104.8	9	5
250	252.4	4	2
500	503.4	4	2
800	778.0	7	4

because in real samples, a competition between analytes, internal standard, and endogenous substances for the active sites on the sorbent backbone cannot be excluded in advance [30]. Differences in interaction between the internal standard and the analytes would result in excessively high or excessively low values, reflecting the extraction selectivity for the standard rather than for the analytes [30]. In fact, when the internal standard was added to the plasma samples, excessively low recoveries were obtained which varied depending on the initial concentration of diltiazem.

The method was first tested in the study of the pharmacokinetic profile of diltiazem in a healthy 25 year old volunteer, after administration of a single 60-mg oral dose of a commercial preparation of diltiazem hydrochloride. Figure 3 shows the chromatogram of an extract of a 1-ml plasma sample obtained 4 h after intake. A pharmacokinetic profile was obtained by the analysis of plasma concentrations at 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 10 h after administration. The time course of the plasma concentration is shown in Fig. 4. The calculated pharmacokinetic parameters were: C_{max} = 63.6 ± 1.7 ng ml^{-1}; T_{max} = 2 h, $T_{½}$ = 5.4 h $AUC_{(1-10)}$ = 260 ng ml^{-1} h. These

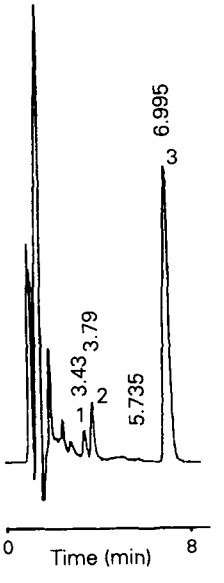

Figure 3
Chromatogram of a plasma sample from a male volunteer 4 h after intake of a single oral 60-mg dose of a diltiazem preparation. Sample clean-up by solid-phase extraction. Peaks: (1) deacetyldiltiazem; (2) diltiazem; (3) verapamil (i.s.). Chromatographic conditions are described in the text. The concentration of diltiazem was calculated as 31 ng ml^{-1}.

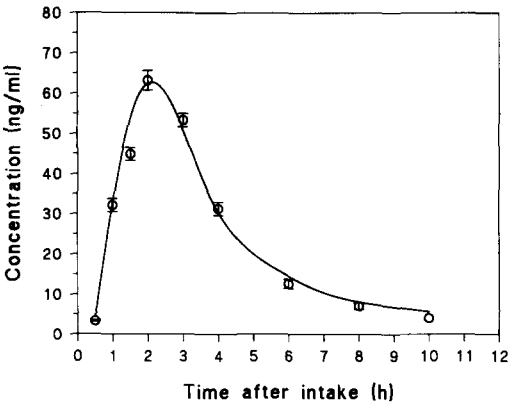

Figure 4
Time course of the plasma concentration of diltiazem following oral administration of a single oral 60-mg dose of diltiazem hydrochloride to a healthy male volunteer.

values are consistent with those obtained by other methods [33, 34].

A randomized blinded study of the pharmacokinetics of three commercial diltiazem preparations in 12 healthy male volunteers was conducted in order to assess the bioequivalency of the preparations. As observed previously [34], there was wide inter-subject variability in the diltiazem concentration indicated by the large standard deviations of the individual average blood concentrations for the same administered preparation as shown in Fig. 5. In most cases, the 24 h concentrations were below the detection limit. Table 2 shows the average C_{max}, T_{max}, $T_{½}$, and AUC for a single oral diltiazem dose of 60 mg. The average plasma concentration–time curves were similar for preparations A and C and somewhat different for preparation B. T_{max} was similar for A and C and shorter than for preparation B. All three diltiazem preparations had similar $T_{½}$ values. The variability of the inter-subject plasma concentrations led to a large variability in the AUC values which precluded making a determination of the bioequivalence of the three diltiazem preparations. This difficulty derives directly from the fact that parametric statistics were applied to data with high variance values. The concept of bioequivalence involves data comparison for classification purposes. However, the individual values from which the pharmacokinetic parameters for each preparation are calculated do not follow a normal distribution. Thus this seems a typical case for the application of multivariate procedures for object classification. For that purpose, each preparation was

Table 2
Pharmacokinetic parameters for three commercial diltiazem preparations A, B and C in a randomized, blind study in 12 healthy male volunteers after oral administration of a single 60 mg dose

Parameter	A	B	C
T_{max} (h)			
Mean	2.4	3.1	2.2
SD	0.6	0.8	0.7
RSD (%)	25	26	32
C_{max} (ng ml^{-1})			
Mean	98	73	87
SD	36	30	31
RSD (%)	37	41	36
$T_{½}$ (h)			
Mean	5.4	6.3	5.4
SD	0.6	1	0.6
RSD (%)	11	16	11
$AUC_{(0-12)}$ (ng ml^{-1} h)			
Mean	455	400	429
SD	118	138	148
RSD (%)	26	35	34
$AUC_{(0-\infty)}$ (ng ml^{-1} h)			
Mean	507	450	478
SD	153	151	167
RSD (%)	30	34	35

considered as an object described by a set of features, in this case C_{max}, T_{max}, $T_{½}$, $AUC_{(0-12)}$ and $AUC_{(0-\infty)}$ mean values, calculated for each preparation for each volunteer. A set of 36 data vectors were obtained, from which one was eliminated as an outlier. Classi-

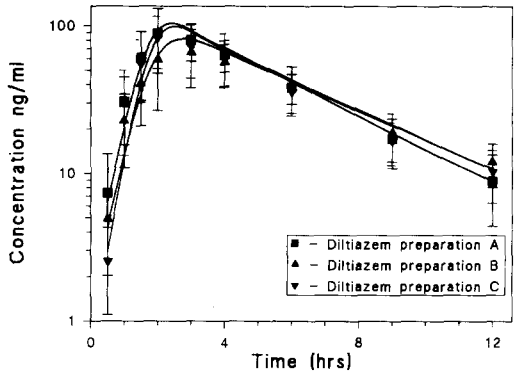

Figure 5
Comparison of average plasma concentration–time curves for three commercial diltiazem preparations after administration of a single oral 60-mg dose to healthy male volunteers.

Table 3
Discriminant function analysis for the classification of three commercial diltiazem preparations by discriminant analysis of the pharmacokinetic parameters

No. of cases for group 1	23
No. of cases for group 2	12
No. of independent variables	5
Variance of discriminant function	2.233657
Degrees of freedom 1	5
Degrees of freedom 2	29
F ratio	3.09579
Discriminant function group 1	−19.56515
Discriminant function group 2	−21.79879
Midpoint cutoff	−20.68197

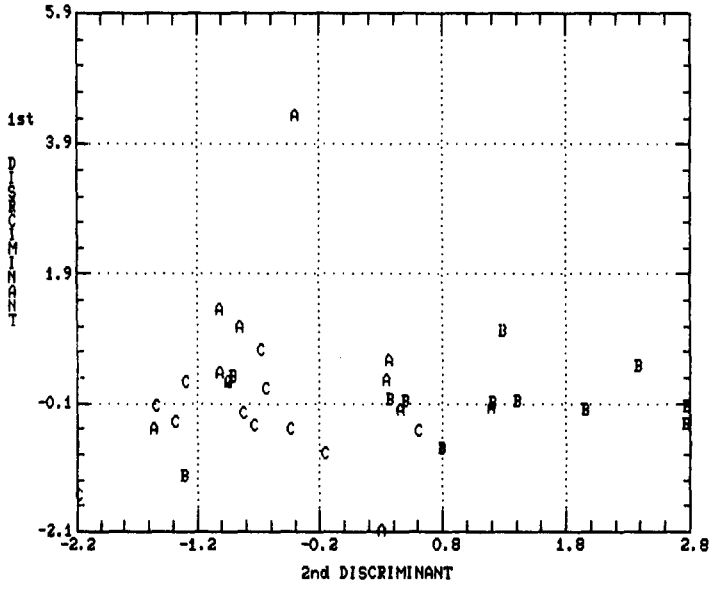

Figure 6
Assessment of bioequivalence of three diltiazem preparations A, B and C by discriminant analysis of the pharmacokinetic data. Plot of the first vs the second discriminant function.

fication of the data vectors into three categories (the diltiazem preparations) by discriminant analysis (Table 3) showed two subsets. One subset group, preparations A and C, clearly separated from the subset corresponding to preparation B. The discriminant scores can be visualized by the plot of the first discriminant vs the second discriminant function as shown in Fig. 6. From group 1 (A and C) only three cases (13%) were misclassified. From group 2 (B) four cases were misclassified (33%). The classification ability of the discriminant function was 80%. Interestingly, from the misclassified samples, three originated from the same volunteer. These results show that classification according to multivariate techniques of data analysis can be used for assessment of bioequivalence between preparations of the same drug from different origins and can complement the classical approach.

The present method for the LC analysis of diltiazem in plasma has several advantages: it is rapid, the limiting factor being the time required for the chromatographic run (about 8 min), since the clean-up process permits the simultaneous work-up of several samples (e.g. 24 samples were processed for prechromatographic clean-up in about 5 min) only a calibration curve is necessary for a concentration range of 10–800 ng ml^{-1}; the assay has a detection limit below the normal range of therapeutic plasma concentrations; and it provides a long column life without loss of performance. In fact, the column used in this study still maintained its analytical capability after more than 6 months of regular use. The pharmacokinetic data can be advantageously used for group classification of pharmaceutical preparations by means of multivariate techniques of data analysis. Discriminant analysis may be a valuable complementary tool in assessing bioequivalence.

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