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High-performance liquid chromatographic assay for diltiazem in small-volume blood specimens and application to pharmacokinetic studies in rats

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed which involves the use of two 5-μm BDS silica gel columns (15 cm × 4.6 mm I.D.) in series for increased resolution and sensitivity, and an organic mobile phase for both extraction and elution of diltiazem. Plasma samples (400 μl) were extracted using the organic mobile phase [*n*-hexane–methanol–dichloromethane–ammonia (370:35:30:0.3)] and the extracts were monitored at 240 nm. Desipramine (30 μg ml^{-1}) was the internal standard. The limit of quantification in plasma was 20 ng ml^{-1} with a correlation coefficient of $\geq$0.999 within the 20–800 ng ml^{-1} standard window. The inter- and intra-assay R.S.D.s were within 5%. The recovery of diltiazem varied from 101.1% at 20 ng ml^{-1} to 93.7% at 400 ng ml^{-1}. The method was applied to the investigation of dilitiazem absorption in a rat. Drug absorption was based on the intestinal single-pass perfusion model. The concentration of diltiazem in all test perfusion solutions was 1 mg ml^{-1} (2.4 m*M*) and the flow-rate through the system was $3.33 \cdot 10^{-3}$ ml s^{-1}. A non-specific mucolytic absorption enhancer was also added to a diltiazem solution and studied in the in situ system. The pharmacokinetics of diltiazem hydrochloride were investigated in two study groups of Wistar rats ($n = 4$). A two-sample Student's *t*-test was employed to compare values of the area under the curve (AUC). The pharmacokinetic data indicated that the AUC in the group which received the enhancer [18.12 ± 5.43 ng ml^{-1} h^{-1} (±S.D.)] was higher than that in the control group (11.49 ± 3.67 ng h^{-1} ml^{-1}), *t*-test; $p = 0.0483$. Hence it was shown that administration of an enhancer could increase the oral bioavailability of diltiazem.

Keywords: Diltiazem; Desipramine

1. Introduction

Diltiazem is a coronary vasodilator or a calcium channel antagonist. It is mainly used in cardiovascular pharmacotherapy for the treatment of angina pectoris, arrhythmia and hypertension. Metabolism of diltiazem in the liver yields the pharmacologically active compounds O-deacetyldiltiazem and N-desmethyldiltiazem (Fig. 1). Previous studies [1,2] in rat, dog and humans have shown that one of the major metabolites in both the liver and intestine was N-desmethyldiltiazem. Several liquid chromatographic (LC) methods have been published for diltiazem [3–15], but none have been reported for the simultaneous determination of diltiazem

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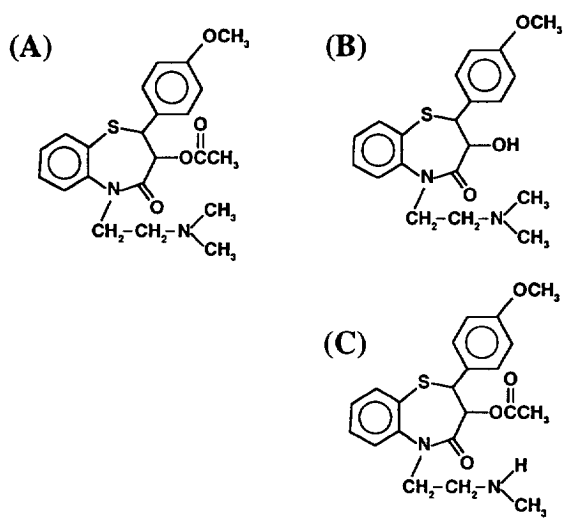

Fig. 1. Structures of (A) diltiazem, (B) deacetyldiltiazem and (C) desmethyldiltiazem.

over a broad standard range (20–800 ng ml^{-1}) in small-volume specimens. A simple, selective and sensitive HPLC method for the determination of diltiazem in plasma is presented here. We have successfully applied this validated method to a pilot bioavailability study in the investigation of diltiazem absorption in a rat. The evaluation of a non-specific mucolytic absorption enhancer is also discussed.

2. Experimental

2.1. Chemicals

Diltiazem hydrochloride was obtained from Vis Farmaceutici (Padova, Italy) and desipramine from Sigma (St. Louis, MO, USA). Hexane, methanol and dichloromethane were supplied by Labscan (Dublin, Ireland) and were of HPLC grade. Ammonia solution (18.1 *M*) was supplied by Riedel-de Haën (Hannover), Germany. Water filtered through a Milli-Q system (Millipore) was used throughout.

2.2. Equipment

The HPLC system consisted of a Model 712 Wisp autosampler, Model 746 data module integrator, Model 486 UV tunable absorbance detector and a Model 501 constant-flow reciprocating pump solvent-delivery system (Waters, Milford, MA, USA). Separation was performed using two 15 cm × 4.6 mm I.D. stainless-steel normal-phase silica (5-μm pore size) columns in series (Waters).

2.3. Chromatographic conditions

The mobile phase was prepared by mixing 0.3 ml of ammonia solution, 35 ml of methanol, 30 ml of dichloromethane and 370 ml of hexane in a flask. The mobile phase was filtered with a 0.45-μm membrane filter and degassed prior to use. It was prepared freshly for each batch of samples. The system was run at ambient temperature. The detector wavelength was set at 240 nm, attenuation = 32 and PT (threshold value) = 200 and the flow-rate was 2 ml min^{-1}.

2.4. Sample preparation

To 400 μl of plasma were added 100 μl of desipramine as internal standard, 100 μl of methanol (volume adjustment) and 500 μl of freshly prepared mobile phase. Each tube was vortex mixed for 30 s and centrifuged at 2000 g for 10 min. The organic layer (200 μl) was carefully transferred into glass injection vials. An injection volume of 100 μl was set on the autosampler.

2.5. Application to a pilot bioavailability study

The method was applied to the investigation of diltiazem absorption in a rat. Drug absorption was based on the intestinal single-pass perfusion model [16].

An isotonic diltiazem solution (300 mOsm l^{-1}, pH 7.2) was administered by perfusion into the ligated intraduodenal segments (ca. 18 cm in length) of pentobarbital-anaesthetized (30 mg kg^{-1}, i.p.) male Wistar rats. The solution was perfused slowly through the isolated duodenal segment at a flow-rate of $3.33 \cdot 10^{-3}$ ml^{-1} for 60 min. The concentration of diltiazem in all test solutions was 1 mg ml^{-1} (2.4 m*M*). For some

rats, the solution also contained a non-specific mucolytic absorption enhancer at a concentration of 0.5% (pH 4.0).

Isolated segments were kept moist by spraying with heated (37°C) isotonic saline. Blood samples (800–1000 μl) were collected from the femoral arteries immediately prior to initiation of perfusion and at 30 and 60 min after commencement of perfusion. Blood sample volume was replaced by administration of isotonic saline.

Blood samples were collected into heparinized tubes and centrifuged at 2000 *g* for 15 min and plasma was harvested for analysis. Samples were stored at −20°C until assay by HPLC.

3. Results and discussion

Typical chromatograms for blank plasma and for plasma spiked with drug are presented in Fig. 2. The retention times for diltiazem and desipramine (internal standard) were ca. 5.9 and 14.0 min, respectively.

Calibration graphs for both within-day and between-day analysis of reproducibility were obtained by plotting the peak-area ratio versus concentration. The calibration graph was linear over the concentration range 0–800 ng of diltiazem per millilitre of rat plasma. The correlation coefficients were ≥0.999 and the linearity of response was determined by weighted least-squares regression analysis of data points. The *y*-intercept was −0.00049 and the slope was 0.00040. The inter- and intra-assay relative standard deviations (R.S.D.s) for the above concentration range were all within 5%. Recoveries in plasma varied from 101.1% at 20 ng ml^{-1} to 93.7% at 400 ng ml^{-1}.

In this HPLC system, the use of the two silica gel columns (in series), an organic mobile phase and UV detection at 240 nm with one-step extraction offers enhanced resolution and sensitivity over a broad calibration range with no interfering peaks from endogenous plasma components. Pre-formulation optimization may be carried out more efficiently, during biopharmaceutics–pharmacokinetic development, owing to the rapid extraction procedure and direct injection of sample on to the chromatographic column. This method has been successfully used in a pilot bioavailability study in which a non-specific mucolytic absorption enhancer was added to a diltiazem solution and studied in situ. Reliable measurements of diltiazem concentrations in plasma were obtained for up to 60 min following intraduodenal administration of dil-

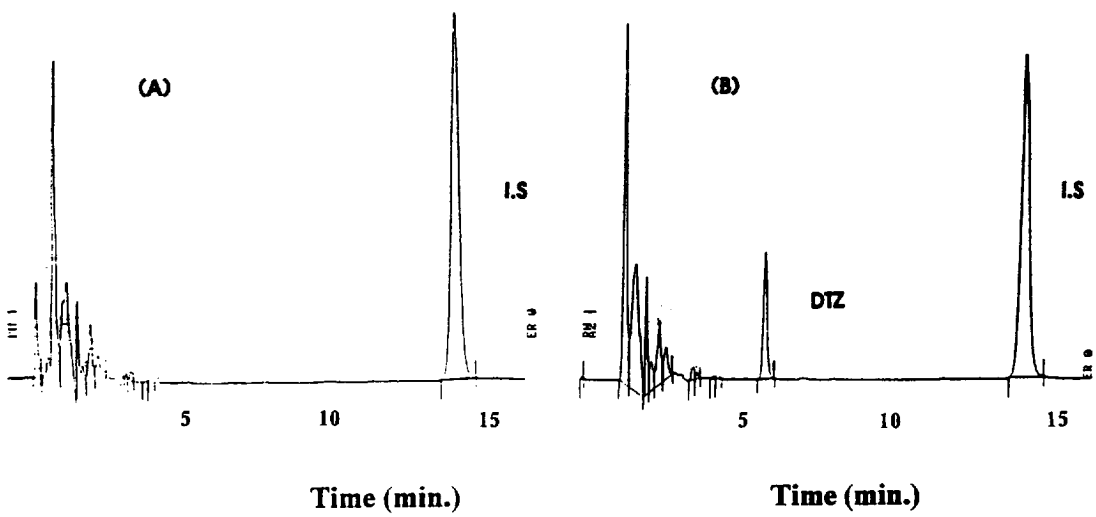

Fig. 2. Chromatograms obtained on analysis of (A) a drug free sample and (B) a 400 ng ml^{-1} extracted plasma standard. Peaks: I.S. = internal standard (desipramine); DTZ = diltiazem.

tiazem (1 mg ml^{-1}) to one group and diltiazem (1 mg ml^{-1}) plus enhancer (0.5%, pH 4.0) to another group.

Pharmacokinetic data were obtained using a two-sample Student's *t*-test to compare the area under the curve (AUC) in the enhancer group [18.12 ± 5.43 ng ml^{-1} h^{-1} ($\pm$S.D.)] with that in the control group (11.49 ± 3.67 ng ml^{-1} h^{-1}), *t*-test; $p = 0.0483$. These results indicate that there is a tendency towards a significantly higher AUC in the group which received the enhancer.

4. Conclusions

This paper has described the first use of a reliable, sensitive and robust method for the determination of diltiazem in plasma. We have demonstrated its application in a pilot animal study where enhancement of absorption was attained following administration of diltiazem with an enhancer via the intraduodenal route. The HPLC method is relatively simple, has good resolution and reproducibility and allows the analysis of 30 samples per day.

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