ABSTRACT:
A simple and sensitive reversed phase high-performance liquid chromatographic method was developed for simultaneous quantification of diltiazem and its major metabolite N-demethyldiltiazem in human plasma. The method involves one-step solvent extraction of diltiazem, N-demethyldiltiazem and the internal standard, verapamil with n-hexane and diethyl ether (50:50 v/v). The mobile phase comprised 0.1M ammonium dihydrogen phosphate-acetonitrile (62:38 v/v) and triethylamine (0.08%) was added before the pH was adjusted to 5.9 with 85% phosphoric acid. Analysis was run at a flow rate of 1.0 ml/mm at a detection wavelength of 238 nm. The completion time for assay was not more than 10 minutes and lower limit of quantification was 5 ng/ml. The calibration curve for diltiazem and its metabolite was linear over a concentration range of 5-200 ng/ml and average recovery was about 90%. The coefficient of variation and percent error values of the assay method within and between day were all less than 10%.

Keywords: Diltiazem; reversed phase HPLC assay.

INTRODUCTION
Diltiazem HCl is a calcium channel blocker currently used in the treatment of angina and also effective in the treatment of hypertension and cardiac arrhythmias. Orally administered diltiazem is metabolized into two basic active metabolites N-demethyl diltiazem and deacetyl diltiazem. The half-life of the metabolite N-demethyl diltiazem was found to be similar to that diltiazem whereas the half-life of deacetyl diltiazem was longer (Hoglund and Nilsson, 1989).

A review of the literature revealed that several high performance liquid chromatographic (HPLC) methods have been reported for the determination of diltiazem and its major metabolites in the plasma. Most of the methods involve liquid-liquid extractions with slight differences in the extraction solvent, mobile phase, the column and the run time for the analysis. Some authors employed reversed phase chromatography with either acid back-extraction (Abernethy et al., 1985; Montamat et al., 1987) or solid-phase extraction procedure (Bouliet et al., 1990) and a normal-phase method (Kinney and Kelly, 1986). A direct injection of plasma samples into the automatic chromatographic system for on-line clean up of plasma samples was also reported by Ascalone and Dalbo (1987).

In this paper, we report a simple, sensitive and specific HPLC method for quantification of diltiazem and its major metabolite in human plasma using ultraviolet detection. The assay method was evaluated for accuracy, precision, recovery and linearity. The method employed a mobile phase of pH 5.9, permitting the use of a cheaper silica-based column. Also, a widely available compound, verapamil was used as the internal standard. The applicability of the method was demonstrated by applying it to analyze plasma samples obtained from a bioequivalence study.
MATERIALS AND METHODS

Materials:
Diltiazem Hydrochloride (Sigma, USA), Verapamil Hydrochloride (USP, Reference standard), N-demethyldiltiazem (Tanabe, Japan), Acetonitrile, HPLC grade (Malinkrodt, USA), Diethyl Ether, AR (BDH, England), N-Hexane, AR (Malinkrodt, USA), Ammonium Dihydrogen Ortho phosphate, AR (BDH, England), Triethylamine, AR (Fluka, Switzerland) and Phosphoric Acid, AR (BDH, England)

Instrumentation:
The HPLC system comprised a Jasco PU-980 Intelligent HPLC pump, a Gilson 119 UV/VIS detector (Gilson Medical Electronics, Villiers-le-Bel, France) connected to a Hitachi D-2500 integrator (Hitachi, Tokyo, Japan) and a Rheodyne 7125 sample injector fitted with a 50 µl sample loop. The detector was operated using a sensitivity range of 0.005 AUFS and wavelength of 237 nm. A LiChrospher 100 RP-18e reversed phase column (5µ/m, 250-x 4.6 mm ID) (Merck, Germany) fitted with a refillable guard column was used for the separation. The mobile phase comprised 0.1 M ammonium dihydrogen phosphate and acetonitrile (62:3 8 v/v). Triethylamine (0.08%) was added before the pH was adjusted to 5.9 with 85% phosphoric acid. Analysis was run at a flow rate of 1.0 mi/mm and the detection wavelength was 238nm with a sensitivity range of 0.005 aufs.

Standard solutions:
Stock solutions were prepared by dissolving 100 mg of diltiazem and N-demethyldiltiazem in 100 ml methanol. The standard curve was prepared by spiking drug free plasma with a known weight of diltiazem and N-demethyldiltiazem at concentration levels of 5, 10, 25, 50, 100 and 200 ng/ml. The standard plasma samples were stored at −20°C in glass bottles.

Extraction Procedure:
A 1.0 ml aliquot of the plasma was accurately measured into a glass tube with a Teflon lined screw cap, followed by addition of 50 µl of internal standard (4 µg/ml of verapamil HCl in aqueous solution and 4.0-ml mixture of diethyl ether and n-hexane (1:1). The mixture was vortexed for 1 mm using a vortex mixer and then centrifuged (Lahofuge 200, Heraeus Sepatech, Germany) at 3500 rpm for 10 minutes. The upper organic layer was transferred into a reactivial (Pierce Reacti-vial, USA) and then evaporated to dryness at 40°C under a gentle stream of nitrogen gas. The residue was reconstituted with 75 µl of mobile phase and 50 µl injected onto the column.

Assay Validation:
Samples were quantified using height ratio of diltiazem or N-demethyldiltiazem over the internal standard. Extraction recovery,
within-day and between-day precision and accuracy studies (n=6) of the method were carried out using plasma standard samples. The recovery of the extraction procedure for diltiazem, N-demethyldiltiazem and internal standard were calculated by comparing the peak height obtained after extraction with that of aqueous solutions of corresponding concentrations without extraction. The accuracy was expressed as percentage error, obtained by calculating the percentage of difference between the measured and the spiked concentration over that of the spiked value, whereas the precision was denoted using the coefficient of variation.

RESULTS AND DISCUSSION

Diltiazem and its metabolites are weakly basic amines and the amino groups are known to interact strongly with the stationary support, causing peak tailing and broadening (Biji et al., 1981; Nahum and Horvath, 1981). Some short-chain tertiary amine modifiers such as triethylamine (TEA) are very effective in improving peak symmetry and reducing retention of solutes with amino functional groups. Therefore, TEA in 0.08% was incorporated into the mobile phase to improve the resolution as well as peak symmetry of diltiazem and its metabolite.

Fig. 1: Chromatograms of (A) blank plasma and (B) plasma sample obtained from a volunteer at 4 hours after oral administration of 90 mg controlled release diltiazem showing peaks for N-demethyldiltiazem (1), diltiazem (2) and the internal standard, verapamil (3).
Simultaneous HPLC quantification of Diltiazem

Chromatograms obtained with blank plasma and plasma sample of a healthy volunteer after 4 h dosing with 90 mg diltiazem are shown in Figure la and lb. The retention times of N-demethyldiltiazem, diltiazem HCl and internal standard (verapamil HCl) were 4.98, 5.78 and 9.88 minutes respectively. The blank sample was clean and no interfering peak was observed at the retention times of above-mentioned components.

The extraction recovery of diltiazem was determined by comparing the peak height obtained by direct injection of standard aqueous solutions to those obtained after the plasma extraction procedure. A mixture of diethyl ether and n-hexane (1:1 v/v) gave better recoveries for N-demethyldiltiazem, diltiazem and internal standard as well as provided cleaner chromatograms compared to using n-hexane, diethyl ether, methyl tertiary butyl ether, ethyl acetate or chloroform alone as the extracting solvent. The recovery of all the components was more than 90%.

The accuracy and precision of the method assessed by analysis of plasma samples at various concentration levels are given in Table 1. The values for the coefficient of variation were all less than 10% at the concentration range determined. The limit of detection was...
approximately 2.5 ng/ml at a signal-to-noise ratio of 3:1. However, the limit of quantification was set at 5 ng/ml being the lowest concentration used in the construction of the standards curve. The mean standard curve of diltiazem only (n=6) is shown in Figure-2. A linear correlation was found between the peak height ratio of diltiazem and the internal standard versus diltiazem concentration in the plasma in the range of 5-200 ng/ml with a regression coefficient of 0.9995. Similarly, linear correlation was observed between the peak height ratio of N-demethyldiltiazem and verapamil versus plasma N-demethyldiltiazem concentration in the range of 5-200 ng/ml.

The method was applied to analyze plasma samples collected from in vivo study of a controlled release test preparation of diltiazem HCl 90 mg. Figure-3 shows the mean plasma profiles of both diltiazem and N-demethyldiltiazem indicating that the concentration of metabolite was greater than drug due to first pass effect and both could still be detected after 24 h after dosing.

CONCLUSION

The present HPLC method is sensitive, simple, specific and appropriate to be used for determination of plasma diltiazem and its major metabolite in pharmacokinetic/bioavailability studies. The pH of the mobile phase used was well within the practical working pH range of common reversed phase C18 columns, which is typically between pH 3-7, thus avoiding the use of more expensive pH sensitive columns. Moreover, the completion time for assay method was less compared to the reported methods.

REFERENCES


