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HPLC Determination of Diltiazem and Deacetyldiltiazem in Rat Plasma

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HPLC를 이용한 랫트 혈장중의 딜타아젬 및 데아세틸딜티아젬의 정량

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A high-performance liquid chromatographic (HPLC) method was developed for the determination of diltiazem (DTZ) and its major metabolite, deacetyldiltiazem (DAD), in rat plasma. DTZ, DAD and imipramine, the internal standard, were selectively fractionated from plasma on a C_{18} reversed-phase column (μ-Bondapak, 10 μm silica, 300×3.9 mm ID). The composition of the mobile phase was methanol: acetonitrile: 0.04 M ammonium bromide: triethylamine (40:24:36:0.06 in volume). The pH of the mobile phase of their method was lowered to 6.4. The eluents from the column were detected for DTZ and DAD using a UV detector at 237 nm. The recovery was >85% for DTZ and DAD, and average intra-day and inter-day coefficients of variation were <6% for DTZ and DAD at the concentration ranges of 20-1000 ng/m*l*. Detection limit of DTZ and DAD in plasma was 20 ng/m*l* with signal-to-noise ratio of 3. This method would be applicable to practical pharmacokinetic studies without detriment to the HPLC column.

Keywords—HPLC, diltiazem (DTZ), deacetyldiltiazem (DAD), pH, mobile phase, sensitivity, inter-day, intra-day variation, recovery

Diltiazem (DTZ) has been widely used as a calcium channel blocking agent[1)] in the treatment of angina pectoris, arrhythmia and hypertension.[2)] It is mainly metabolized in the body to deacetyldiltiazem (DAD).[3)] For the asay of DTZ and DAD, various methods including thin-layer chromatography (TLC),[4)] gas chromatography (GC),[3,5,6)] and high performance liquid chromatography (HPLC)[7-15)] have been reported. Most of HPLC methods are more sensitive than TLC and GC methods. Ion-pairing HPLC by Goebel and Kolle[15)] is one of the most sensitive methods for DTZ and four metabolites including DAD in the plasma, ie., its detection limits were 0.1 and 0.2 ng/m*l* for DTZ and DAD in plasma, respectively. However, the pH of mobile phase of this method was maintained at 8.5, which is detrimental to C_{18}-bonded silica column.[16,17)] We report a modified HPLC method, which is not detrimental to the column, for the simultaneous assay of DTZ and DAD in the rat plasma. The sensitivity, inter-day and intra-day variation, and reproducibility of the present method were also examined.

Experimental

Chemicals and Animals

DTZ and DAD were kindly given by Han-Il

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Pharmaceutical Co. (Seoul, Korea). Heparin of 5,000 IU/m*l* was purchased from Choong-Wae Pharmaceutical Co. (Seoul, Korea). Imipramine (IMP), an internal standard in HPLC, was given by Hwan-In Pharmaceutical Co. (Seoul, Korea). Methanol (Merck), acetonitrile (Merck) and triethylamine (Aldrich) were of HPLC grade. All other reagents were of analytical grade and used as purchased.

Male Wistar rats weighing 200-260 g were from the Experimental Animal Center of Seoul National University.

Preparation of Calibration Curves of DTZ and DAD in Rat Plasma

Stock solutions (1 mg of free base/m*l*) of DTZ, DAD and IMP were prepared in methanol. Standard solutions were made by sequential dilutions of the stock solution with methanol from 20 to 1000 ng/m*l* for both DTZ and DAD, and 2 μg/m*l* of IMP. All solutions were stored at 4℃ until used. DTZ and DAD were found to be stable for at least 3 months, and IMP for 6 months, respectively.

Fifty microliters of the standard solutions in a 15 m*l* polypropylene tube was evaporated to dryness with a gentle stream of nitrogen. An aliquot of 100 μ*l* of plasma sample was added to the residue and vortexed. The sample was then extracted with 3 m*l* of *t*-butyl methyl ether by vortexing for 5 min. After centrifugation, the tubes were placed in a dry ice bath and 2.5-m*l* aliquots of the unfrozen upper organic phase were transferred to other tubes. Subsequently the organic layer were back-extracted with a 100 μ*l* of 0.01 N HCl by vortexing for 1 min. Aliquots of 30 μ*l* were taken from the HCl layer and injected into HPLC system.

Assay of DTZ and DAD in Plasma Samples after DTZ Administration

Six rats were fasted 24 hr before the experiment. The rats were fixed at supine position during the experiment. Under light anesthesia, the femoral artery and veins of the rats were cannulated with polyethylene tubing (PE-50). After complete recovery (1 hr) from anesthesia, a 0.3% (w/v) DTZ solution in saline was administered intravenously to the femoral vein through the catheter at a dose of 1 m*l*/kg (3 mg/kg). Blood samples (250 μ*l*) were withdrawn into heparinized tubes from the femoral artery catheter at 0, 5, 10, 20, 30, 45, 60, 90, 120, 150, and 180 min after dose. Plasma samples were separated by centrifuging the blood samples at 6000 g for 1 min and were stored at −20℃ until HPLC assay for DTZ and DAD. DTZ and DAD in plasma were assayed as follows. Fifty microliters of the internal standard solution (2 μg/m*l* of IMP in methanol) in a polypropylene tube was evaporated to dryness with a gentle stream of nitrogen. The procedure afterwards was identical to that for the calibration curve study.

HPLC

The HPLC system consisted of a precision isocratic pump (Model SP 8010, Spectra-Physics), a C_{18} reversed-phase column (μ-Bondapak, 10 μm silica, 300×3.9 mm ID, Waters), a C_{18} reversed-phase guard column (30 μm, Alltech Associates, Inc.) and a UV absorbance detector (Model 757, Applied Biosystems). The mobile phase was a mixture of methanol, acetonitrile, 0.04 M ammonium bromide and triethylamine (40:24:36:0.1 volume ratio). The pH of the mobile phase was adjusted to 6.4 using 2 N hydrobromide. The flow rate of the mobile phase was 1.0 m*l*/min and the detector was set at 237 nm. The ratio of peak heights of DTZ or DAD against IMP were used for the quantitative calculation of each compound in the plasma throughout the study.

Assay Validation

Intra-day variation was measured by assaying plasma samples of seven different concentrations ranging between 20-1000 ng/m*l* triplicately per each sample in a day. Inter-day variation was measured by assaying the same samples once in a day for consecutive three days.

In order to determine the recovery of the present method, 100 μ*l* of drug-free plasma was spiked with varying amounts of DTZ and DAD and constant amount of IMP (100 ng). The spiked amount was varied from 20 ng to 100 ng for DTZ and DAD. The recovery of each compound was calculated as follows:

Recovery (%)=(peak height from the plasma sample)/(peak height from the standard solution of the same concentration)×100 (1)

Results and Discussion

Fig. 1 shows the HPLC peaks of DTZ, DAD and IMP in the plasma samples under the given condition. DTZ, DAD and IMP were sharply separated and the retention times were 5 min for DAD, 6 min for DTZ, and 7 min for IMP. The present method is basically similar to the method of Goebel *et al.*[15] in respect to the composition of the mobile phase, but the pH of the phase was lowered from 8.5 to 6.4 in this study. Si-C bond of the C_{18} column material is unstable to the mobile phase of pH >7 containing aqueous salt solution especially at higher temperatures.[19] Actually, the mobile phase composition of methanol: acetonitrile: 0.04 M ammonium bromide: triethylamine (40:24:36:0.06 in volume) with a pH of about 8.5[15] gave a detrimental effect on μ Bondapak column within 24 hr of its usage. Therefore, the pH of the mobile phase was adjusted to 6.4 by adding 2 N HBr solution in this study. Bromide ion forms the ion-pair complexes with DTZ, DAD and IMP in the mobile phase.[15]

The retention time was very sensitive to the pH of the mobile phase. At pH >6.5, the peaks of DTZ and IMP tended to be folded together and at pH <6.3, the peak of DAD tended to be immersed in the peaks of the blank plasma. Therefore, adjustment of pH of the mobile phase at pH 6.4 seemed to be critical to separate DTZ, DAD and IMP.

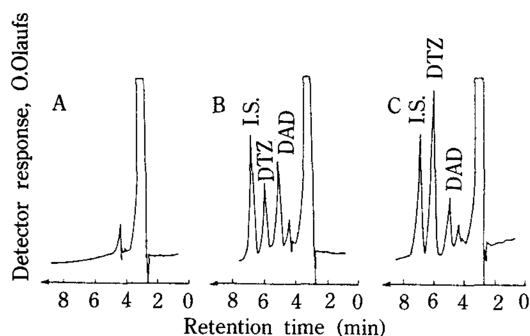

Figure 1—Typical chromatograms of the blank plasma (A), plasma spiked with DTZ and DAD (200 ng/m*l* of each) (B), and plasma collected 20 min after intravenous injection of DTZ (3 mg/kg dose) (C). I.S., an internal standard, was added to B and C to yield its concentration of 1000 ng/m*l*.

The calibration curve study showed very good linearity for both DTZ ($r=0.9997$, $p<0.00001$) and DAD ($r=0.9995$, $p<0.00001$) in the concentration range of 20-1000 ng/m*l*. The detection limit of the assay for both DTZ and DAD was 20 ng/m*l* with signal-to-noise ratio of 3 and the coefficient of variation (CV) of <10%.

Table I shows the intra- and inter-day CV of the present assay. In the concentration range of 20-1000 ng/m*l* plasma, it was kept less than 10%. Table II shows the recoveries of DTZ and DAD.

Table I—*Intra-day and Inter-day Reproducibility for DTZ and DAD*[a]

Concentration	Intra-day CV (%)		Inter-day CV (%)	
(ng/m*l*)	DTZ	DAD	DTZ	DAD
20	8.7	1.8	6.5	9.0
50	8.4	4.7	8.9	7.1
100	6.5	1.6	5.6	1.8
200	3.5	4.3	6.6	5.7
500	2.5	5.2	2.3	5.1
800	4.0	3.2	2.4	4.7
1000	2.5	4.7	2.3	2.4
Mean	5.2	3.6	4.9	5.1
(± SD)	(2.7)	(1.5)	(2.6)	(2.5)

[a]Each CV was calculated with three determinations.

Table II—*Recovery of DTZ and DAD from Plasma Samples*[a]

Concentration	Recovery (%)	
(ng/m*l*)	DTZ (n=3)	DAD (n=3)
20	86.7 (8.7)	86.2 (1.8)
50	86.3 (8.4)	92.0 (4.7)
100	92.4 (6.5)	97.1 (1.6)
200	92.6 (3.5)	96.2 (4.3)
500	94.0 (2.5)	97.6 (5.2)
800	93.4 (4.0)	96.3 (3.2)
1000	91.7 (2.5)	97.1 (4.7)
Mean	91.0	94.6

[a]Each value was expressed as mean (± SD) of three determinations.

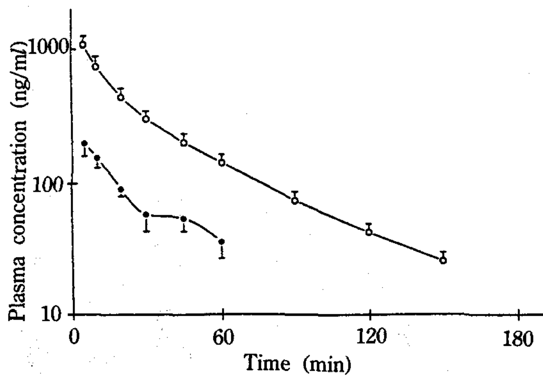

Figure 2—Plasma concentration-time profiles of DTZ (○) and its major metabolite (●) after intravenous injection of DTZ at a dose of 3 mg/kg to rats. Each point represents mean± SD of six experiments.

They were >85% for DTZ and DAD.

Fig. 2 shows the representative plasma profiles of DTZ and DAD measured with the present method after the intravenous administration of DTZ to six rats at a dose of 3 mg/kg. Plasma disposition of DTZ showed 2-exponential decay with very rapid formation of DAD. The pharmacokinetics of DTZ in the rat studied using the present HPLC method will be presented elsewhere.[18]

In short, the present method will enable the *simultaneous* assay of DTZ and DAD without detriment to the column, which was inevitable in the original method of Goebel and Kolle.[15] The assay sensitivity, intra-day and inter-day variation, and recovery were almost comparable to the method of Goebel and Kolle,[15] and seem to be enough for the pharmacokinetic studies of DTZ and DAD in rats.

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