

High Performance Liquid Chromatography of Mebudipine: Application to Pharmacokinetic Study

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Abstract. PURPOSE: To develop a high performance liquid chromatography system for the determination of a new 1,4-dihydropyridine, mebudipine, in rabbit plasma. **METHODS:** To 1 ml of rabbit plasma was added internal standard (dibudipine) and 0.5 ml of 1 M NaOH. Mebudipine and internal standard were extracted to 5 ml ethyl acetate, evaporated under slow stream of nitrogen. The residue was reconstituted in 200 μ l mobile phase and 20 μ l of aliquots were injected into a HPLC system equipped with 4.6 \times 250 mm i.d. C₁₈ analytical column. Mobile phase consisted of methanol (70%), water (25%) and acetonitril (5%) and its flow rate was 1 ml/min. **RESULTS:** There were no interfering peaks from endogenous components in blank plasma chromatograms. Standard curves were linear ($r^2 > 0.99$) over 10 to 500 ng/ml. The extraction efficiency was $>90\%$ and the minimum quantifiable concentration was 10 ng/ml (CV $<10\%$). **CONCLUSION:** A suitable, convenient and simple HPLC assay for pharmacokinetic study of mebudipine in rabbits was developed.

INTRODUCTION

Mebudipine [(\pm)-t-butyl, methyl-1, 4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylate] is a new calcium channel blocker with 1, 4-dihydropyridine structure (Figure 1) that was first synthesized by Mahmoudian *et al* in our laboratory (1). In previous studies it was shown that mebudipine had comparable pharmacological effect with nifedipine while offering some advantages such as longer biological half life, longer time to reach peak effect and more vasoselectivity (1, 2). This paper describes an assay method for mebudipine in plasma and its application to a preliminary pharmacokinetic study was conducted to demonstrate the usefulness of the method.

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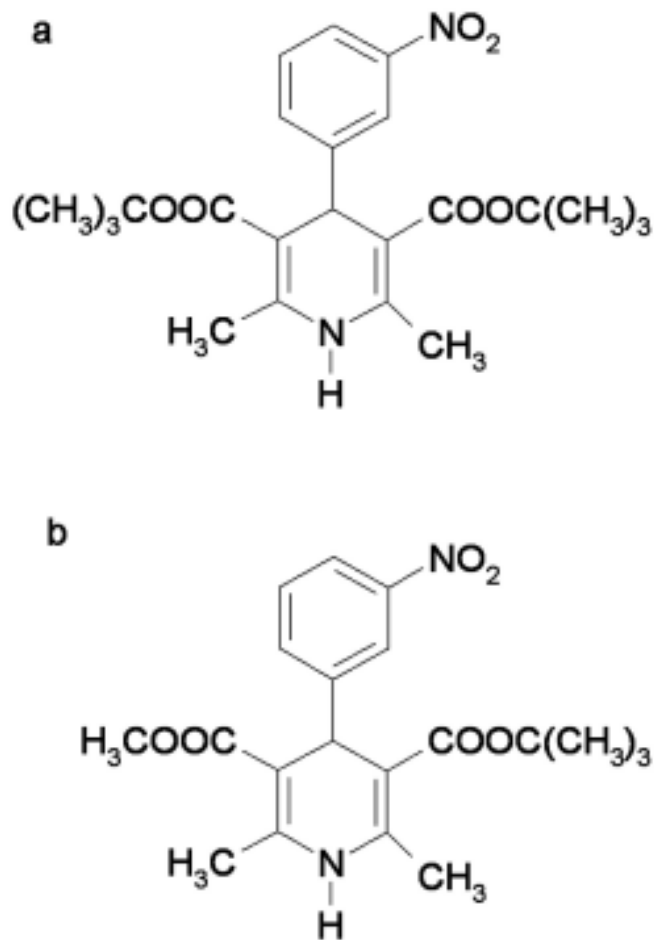


Figure 1: Chemical structure of a) dibudipine and b) mebudipine.

MATERIALS AND METHODS

Chemicals

Mebudipine and dibudipine (internal standard, Figure 1) were synthesized in our laboratories as described previously (1). HPLC grade methanol, acetonitril and ethyl acetate were purchased from Merck (E. Merck, 64271 Darmstadt, Germany). All other reagents were analytical grade.

HPLC System

The HPLC system consisted of a Waters liquid chromatography system (Waters chromatography divi-

sion. Milford, MA, USA) including a model 600 controller, a model 600 pump, a model 486 UV tunable absorbance detector, a model 7725i Rheodyne manual injector and a model 746 data module. An analytical column (Novapack ODS, 5 μm , 4.6 mm X 250 mm) and a guard column (C18 Waters, MA, USA) were used for all analyses. The mobile phase consists of methanol-water-acetonitril (70-25-5). The chromatography analyses were performed at ambient temperature at flow rate of 1 ml/min and the elute was monitored at 238 nm.

Standard Solution

Stock solution of mebudipine and dibudipine were prepared in methanol (1mg/ml), protected from light and stored at -20° until used. Solutions of 20 and 2 $\mu\text{g}/\text{ml}$ were prepared by diluting the stock solution in methanol. The stability of these solutions was compared against a freshly prepared stock solution. Standard curves for plasma were prepared by adding known amount of mebudipine and dibudipine to pooled blank plasma followed by HPLC analysis. Peak-height ratios of samples were used to calculate mebudipine plasma concentration from standards.

Sample preparation

To one ml of plasma sample were added, 10 μl of internal standard (dibudipine, 20 $\mu\text{g}/\text{ml}$) solution and 0.5 ml of 1M NaOH. The solution was mixed for a few seconds. Five ml ethyl acetate was added to the solution which was subsequently shaken on horizontal shaker for 10 min followed by centrifugation at 18000 g for 10 min. The organic layer was transferred to a clean glass tube and evaporated to dryness under nitrogen at 40° . The resultant residue was reconstituted with 200 μl of mobile phase and 20 μl was injected into the HPLC.

Extraction efficiency

Different solid phase extraction methods were examined and were found to be inappropriate due to low extraction efficiency. In addition various organic solvents were tested for the extraction procedure and ethyl acetate proved to be the most suitable because of smaller interference of endogenous components and good extraction efficiency. The extraction efficiency was calculated by adding known amount of mebudipine (10, 50, 200 and 400 ng/ml, n=4) to 1 ml rabbit

plasma. Mebudipine was extracted as described above. The peak heights of mebudipine from spiked plasma samples were compared with the peak heights obtained after direct injection of 20 μl of 10, 50, 200 and 400 ng/ml mebudipine solutions.

Accuracy and Precision

For the determination of intra-day and interday accuracy and precision of the assay, aliquots of 1 ml rabbit plasma were spiked with 10 μl of 20 $\mu\text{g}/\text{ml}$ of internal standard and various quantities of mebudipine to yield 10, 50, 200 and 400 ng/ml. Accuracy was expressed as the mean% [(mean measured concentration)/(expected concentration)] $\times 100$ (3). Precision was calculated as inter and intra-day coefficient of variation [%CV=(SD/mean) $\times 100$] (3).

Pharmacokinetic of Mebudipine in Rabbits

Three adult male albino rabbit were each administered single bolus intravenous doses of 0.50 mg/kg mebudipine dissolved in 60% PEG 400). Blood samples were collected from marginal ear vein at 5, 10, 20, 30, 60, 120, 180, 240 min after mebudipine administration. Plasma was separated by immediate centrifugation and was kept at -20° until analyzed. Pharmacokinetic parameters were calculated by two-compartmental method (4) using DRUG-KNT program (5).

RESULTS AND DISCUSSION

Acetonitril was used for denaturing of plasma proteins. Denaturation with acids reduced the assay's extraction, due perhaps to composition of the drug.

We tried solid phase method for extraction procedure. Initially a silica-based solid extraction cartridge was used which gave clean chromatograms. However, the method was not reproducible and the extraction efficiency was less than 50%. Consequently, a C₁₈ extraction cartridge was employed. Mebudipine was eluted with different organic solvents from solid phase. Best result was produced when cartridge was washed with 6 ml pH 8 phosphate buffer in three 2 ml steps followed by a 3 ml acetonitril wash. Under these conditions, the assay was reproducible but the extraction efficacy was still less than 60%. Liquid phase extraction method yielded the best results. Ethyl acetate showed good

extraction efficiency (>90%) and clean blank chromatograms.

Typical chromatograms of blank plasma, spiked plasma and an actual sample obtained from the pharmacokinetic study are shown in Figure 2.

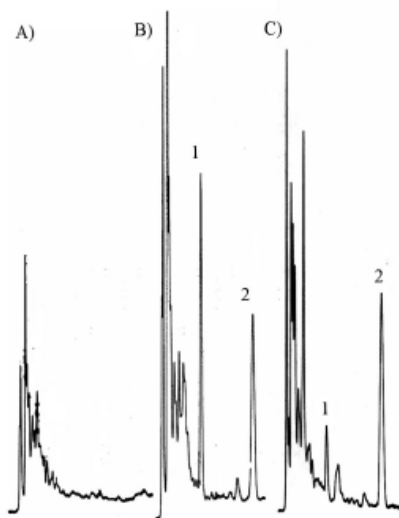


Figure 2: Chromatograms of mebudipine in rabbit plasma. A.) blank rabbit plasma, B.) sample spiked with 50 ng/ml mebudipine and 200 ng/ml IS, and C.) sample obtained 20 min after an IV dose of 500 µg/ml mebudipine. Peaks: 1=mebudipine 2=dibudipine.

Retention time of mebudipine and internal standard were approximately 8 and 15 min, respectively. The total HPLC run time for each sample was about 20 min. There were no interfering peaks in the blank plasma samples. Standard curves prepared for mebudipine in rabbit plasma was linear over 10 to 500 ng/ml. The mean (n=3) calibration curve for mebudipine was $y=0.008x - 0.0022$, $r^2=0.9989$ where, y and x are the peak height ratio and concentration (ng/ml), respectively. Mebudipine concentration as low as 10 ng/ml could be quantified. The sensitivity of assay was sufficient for pharmacokinetic study. The accuracy of assay was >90% and CV did not exceed 10% (Table 1). The mebudipine stock solution was stable at least for 3 months when the solution was protected from light and kept in -20°. However, when stock solutions were kept in the room temperature for approximately after two weeks, several peaks representing degradation products appeared in chromatograms.

Table 1: Intra and interday variation of mebudipine assay in rabbit plasma.

Prepared Concentration (ng/ml)	Measured Concentration (ng/ml)	C.V. (%)	Accuracy (%)
Intraday			
10	9.78	6.66	97.82
50	45.55	4.57	91.1
200	188.67	3.26	94.34
400	402.34	7.3	100.58
Interday			
10	10.27	9.6	102.73
50	50.41	9.7	100.83
200	198.62	2.53	99.3
400	391.2	6.7	97.8

The method mentioned above was applied to pharmacokinetic study of mebudipine in rabbits. The mean plasma concentration time curve is shown in Figure 3 while corresponding pharmacokinetic parameters are reported in Table 2.

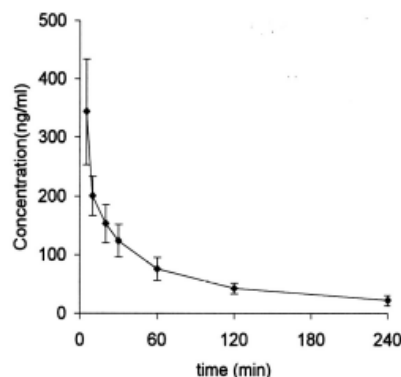


Figure 3: Mean mebudipine plasma concentration-time profile in rabbits following IV administration of 500 µg/kg mebudipine. Each point represents the mean ± SE for three rabbits

Table 2: Pharmacokinetic parameters of mebudipine following intravenous bolus administration of 0.50 mg/kg mebudipine in three rabbits.

Animal	AUC ₀₋₄	AUC _{0-∞}	t _{1/2}	CL _T	V _{ss}
R1	176	214	2.03	5.31	6.8
R2	342	445	2.16	1.9	3.5
R3	177	214	2.02	5.31	5.73
Mean±SE	231±78	291±109	2.07±0.06	4.18±1.6	5.34±1.37

AUC, area under plasma concentration-time curve; CL_T, total body clearance; V_{ss}, steady-state volume of distribution

After IV administration, plasma concentration curves were bioexponential. The α and β phase declined with a mean half-life of 16 min and 134 min respectively. The total clearance of mebudipine was 4.2 l/h/kg. The volume of distribution at steady state of 5.34 l/kg indicates that mebudipine distributed into tissues to a moderate extent due, perhaps, to its lipophilic nature.

CONCLUSION

The HPLC assay presented here is suitable for pharmacokinetic study of mebudipine in rabbits. This assay is convenient, simple and sensitive.

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