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Short communication

High-performance liquid chromatographic analysis of mibefradil in dog plasma and urine

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Abstract

The objective of the study was to develop a sensitive and specific assay for studying the pharmacokinetics of a novel calcium antagonist, a benzimidazolyl-substituted tetraline derivative, mibefradil (I) in the dog. The assay involves liquid–liquid extraction of a biological sample, reversed-phase HPLC separation and fluorescence detection (λ_{ex} = 270 nm and λ_{em} = 300 nm) of sample components. Each sample was eluted with a mobile phase pumping at a flow-rate of 2 ml/min. The mobile phase composition was a mixture of acetonitrile and aqueous solution (38:62, v/v). The aqueous solution contains 0.0393 *M* KH_2PO_4 and 0.0082 *M* Na-pentanesulphonic acid. The retention times were 10.7 min for I, and 12.2 min for internal standard Ro 40-6792. Calibration curves with concentrations of I ranging from 10 to 500 ng/ml were linear ($r^2 > 0.99$). The detection limit for I was 0.5 ng/ml when 0.5 ml of plasma or urine was used. Intra- and inter-day accuracy and precision were within 10%. The assay was successfully applied to the pharmacokinetic studies of I in dogs.

1. Introduction

Calcium channel blockers are widely used therapeutic agents for the treatment of various cardiovascular ailments. They are one of the standard first-line treatments for essential hypertension. In combination with β-blockers and nitrates, they have become established therapy for angina pectoris [1]. Verapamil, diltiazem and nifedipine are the best known prototypes of the first-generation calcium channel blockers. Their main pharmacokinetic and pharmacodynamic characteristics are low bioavailability and relatively short duration of action. In recent years much effort was oriented towards developing new agents with improved kinetic and dynamic profiles. Mibefradil (1), a benzymidazolyl-substituted tetraline derivative, is one of these novel calcium antagonists. The structure of this drug, (1*S*,2*S*)-2-[2-[[3-(2-benzimidazolyl) propyl] methylamino]ethyl]-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl methoxyacetate dihydrochloride, is shown in Fig. 1. Pharmacokinetic and pharmacodynamic studies in animals [2,3,4] and humans [5] have shown promising results and I is currently in the phase III clinical development. Dose escalating studies in humans showed that pharmacokinetics after intravenous administration appears to be independent of the dose. Oral dose studies demonstrated decrease in oral clearance and increase in bioavailability with increasing dose and multiple doses. It is

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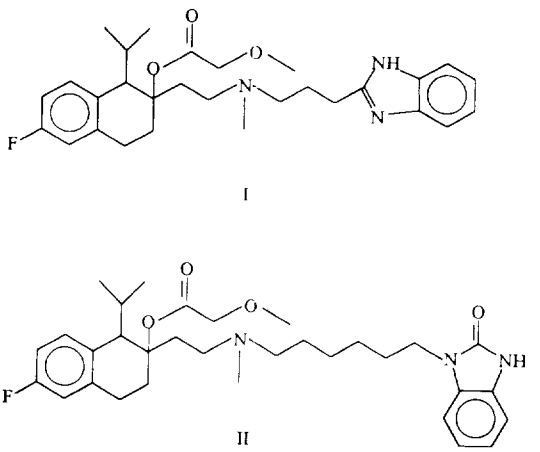

Fig. 1. Structures of mibefradil (I) and internal standard (II).

important to understand the underlying mechanism of this phenomenon which is important in drug therapy design. We intended to initiate a series of experiments to evaluate the applicability of dogs as a model for human kinetics. A sensitive and specific HPLC assay for measuring I in plasma and urine samples was therefore developed. It is the objective of this paper to report the assay used in our animal studies.

2. Experimental

2.1. Chemicals

The hydrochloride salt of I and internal standard (Ro 40-6792, II), an analogue of I, were kindly supplied by Hoffmann–La Roche (Basel, Switzerland). The structures of I and II are shown in Fig. 1. Solvents, acetonitrile and *tert.*-methylbutyl ether, were HPLC grade (Fischer Scientific, Montreal, Canada). Other chemicals such as KH_2PO_4, $KHCO_3$ (BDH, Toronto, Canada) and Na-pentanesulphonic acid (Sigma, St. Louis, MO, USA) were analytical grade.

2.2. Standard solutions

Standard stock solutions of I were prepared in deionized water to provide final concentrations of 5 and 10 μg/ml base equivalent. The concentration of the internal standard solution was 8 μg/ml in deionized water. Drug-free dog plasma and urine samples were spiked with the appropriate stock solution of I. Serial dilutions with the appropriate biological matrix gave standard concentrations ranging from 10 to 500 ng/ml in plasma and 10 to 200 ng/ml in urine. Stock solutions of I and internal standard were prepared every 3 months and stored at −25°C; working solutions were kept at 4°C for a period of a month.

2.3. Sample preparation

A 0.5-ml volume of saturated solution of $KHCO_3$ and 50 μl of internal standard solution were added to the 0.5 ml aliquots of plasma and 0.2 ml aliquots of urine. Aliquots of 0.25 ml plasma of samples exceeding the standard curve range were diluted with blank plasma to the volume of 0.5 ml. Each sample was extracted with 2 ml of *tert.*-methylbutyl ether by vortexing for 30 s and centrifuged at 1000 *g* for 3 min. Bottom water phase was frozen on an acetone/dry ice bath and the organic phase was transferred into clean tubes (Kimax, Kimble, IL, USA) and evaporated under vacuum (Savant Instruments, Farmingdale, NY, USA). The residue was reconstituted in 200 μl of mobile phase and 30–130 μl of this solution was injected onto the HPLC system. The extraction efficiency was found to be higher than 90% for I.

2.4. Instrumentation and chromatographic conditions

The HPLC system consisted of a SIL 9A automatic injector (Shimadzu), a Model 501 pump, a Model 470 scanning fluorescence detector and IBM-compatible PC with a Baseline 810 data processing software (Waters, Mississauga, Canada). The detector was set at $\lambda_{ex} = 270$ nm and $\lambda_{em} = 300$ nm and chromatographic separation was achieved on a reversed-phase column (Merck, LiChrospher, C_{18}, RP Select-B). The mobile-phase composition was a mixture of acetonitrile and aqueous solution (38:62, v/v). The

aqueous solution contains 0.0393 *M* KH_2PO_4 and 0.0082 *M* Na-pentanesulphonic acid. Mobile phase was pumped at a flow-rate of 2 ml/min.

2.5. Calibration curves and assay validation

Calibration curves were generated by weighted ($1/c$) least-squares regression of the analyte/internal standard peak-height ratio vs. the concentration of the analyte. The assay was validated using quality control (QC) samples prepared by an authorized person in the laboratory. The concentration of these QC samples covered the range of the calibration curve. Intra-assay accuracy and precision were assessed from replicate analyses ($n = 3$) of spiked plasma at four different concentrations (10, 30, 200 and 500 ng/ml) on the same day. Three replicates were performed for urine at the concentrations of 10, 30 and 200 ng/ml. Inter-assay validation for

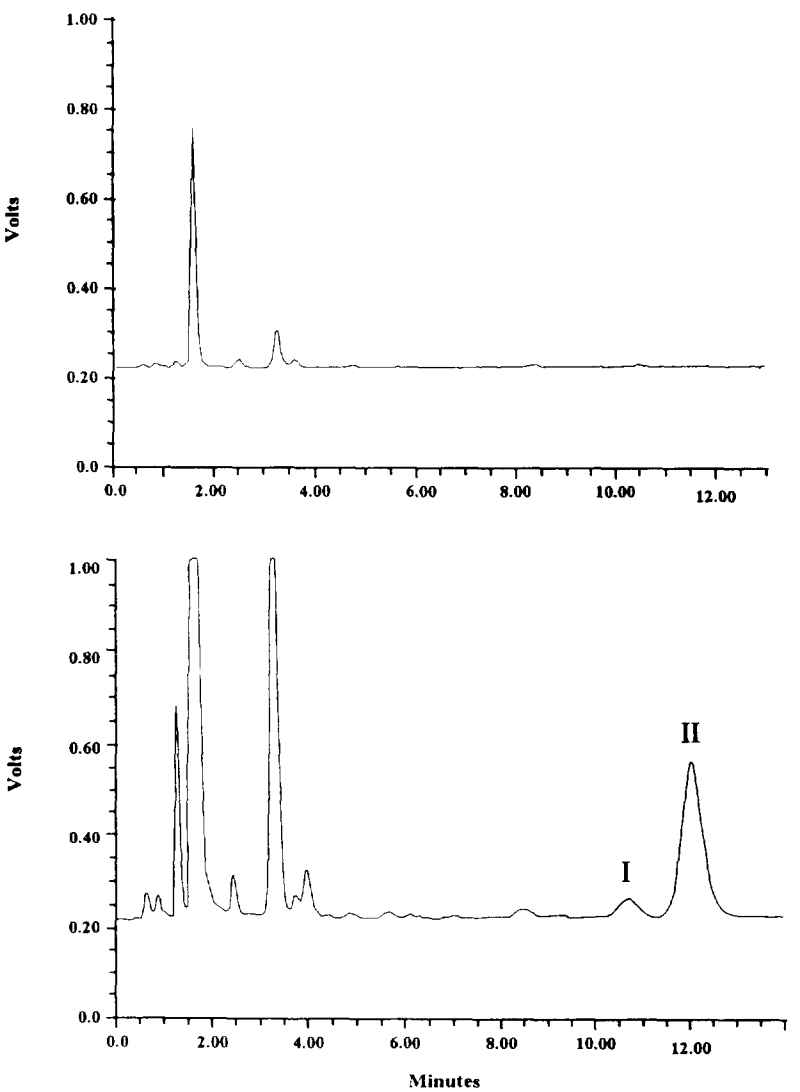

Fig. 2. Representative chromatograms of blank dog plasma (upper panel) and dog plasma taken at 36 h after a 3 mg/kg single oral dose of I (lower panel). The concentration of I corresponds to 12 ng/ml.

plasma and urine was performed on three separate occasions.

3. Results and discussion

Representative chromatograms are shown in Figs. 2 and 3. The retention time of I was 10.7 min while the internal standard was eluted at 12.2 min. Using 0.5 ml of plasma and 0.2 ml of urine, calibration curves with concentrations ranging from 10 to 500 ng/ml for plasma and 10 to 200 ng/ml for urine were linear ($r^2 > 0.99$). The regression equations in plasma and urine were $y = 190x + 1.4$ and $y = 188x + 1.1$, respectively. The detection limit for I was 0.5 ng/ml for both biological media. Dilution of urine samples was not required since the urinary excretion of I in dogs is less than a 1% of the dose after intravenous and oral administrations. Assay per-

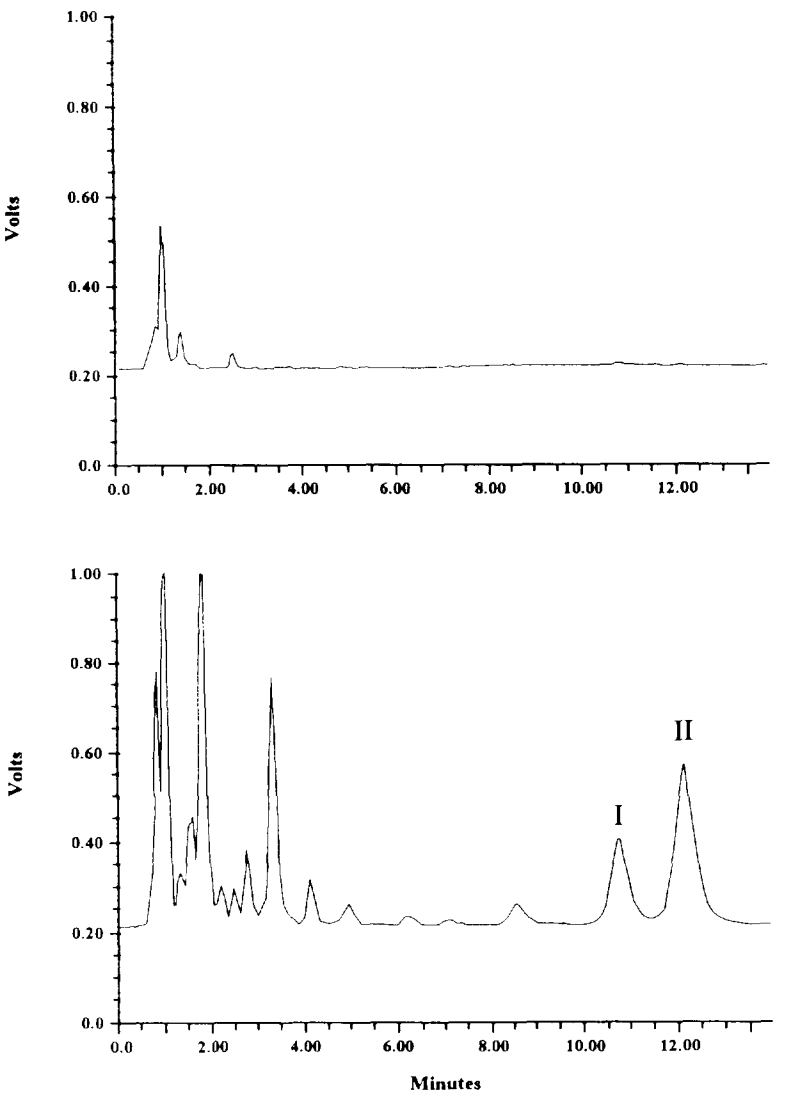

Fig. 3. Representative chromatograms of blank dog urine (upper panel) and dog urine collected between 12 and 24 h after a single oral dose of 6 mg/kg of I (lower panel). The concentration of I corresponds to 64 ng/ml.

Table 1
Accuracy and precision of the assay for I in plasma (PL) and urine (UR)

Concentration added (ng/ml)	Intra-day ($n = 3$) Accuracy (%)		Precision (C.V., %)		Inter-day ($n = 3$) Accuracy (%)		Precision (C.V., %)	
	PL	UR	PL	UR	PL	UR	PL	UR
10.0	105.7	103.9	2.1	3.1	109.1	95.8	5.5	6.0
30.0	94.7	101.3	6.1	4.6	94.7	99.0	6.1	5.0
200.0	103.9	96.0	3.1	4.0	96.0	97.0	3.2	6.1
500.0	104.5		2.1		103.0		1.2	

formance during routine analysis was evaluated using quality control samples resulting in similar accuracy and precision for both biological media (Table 1).

Currently, little information is available on the metabolism of I in dogs. Metabolism in rats has been intensively investigated [6] and appears to be complex, giving rise to a multitude of products. Major metabolic pathways include *N*-demethylation, hydrolysis of the ester side chain, hydroxylation, aromatization of the tetrahydronaphthyl system, loss of benzimidazole and glucuronidation of hydroxyl groups. The resultant metabolites are predominantly excreted into the bile. It is reasonable to expect similar metabolic reactions to occur in dogs. Although the extent of biliary excretion of metabolites in dogs is unknown, our results show that some of the metabolites appear in systemic circulation (Figs. 2 and 3). These unknown metabolites do not interfere with quantification of I in plasma and urine. Studies are underway to identify and quantitate these metabolites.

The method presently described proved to be selective, sensitive and simple. The assay has been successfully applied to several hundreds of samples from our dog studies and representative plasma vs. time profiles are illustrated in Fig. 4.

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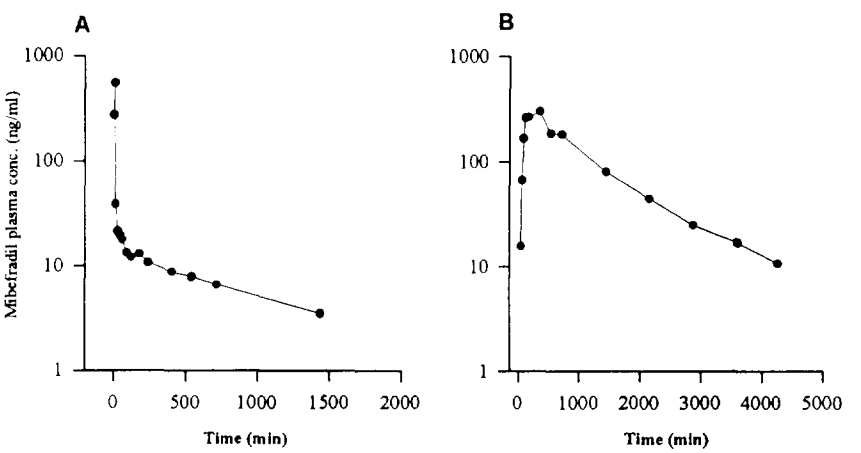

Fig. 4. Plasma concentration vs. time profile in a representative dog (female, cross-bred), weighing 22 kg: (A) after a 10 min intravenous infusion of 1 mg/kg of I and (B) after a single 6 mg/kg oral dose of I.

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