Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 7, pp. 565–571, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00
Pergamon Press plc

Liquid chromatography assay for amlodipine: chemical stability and pharmacokinetics in rabbits*

POLLEN K.F. YEUNG,† SUSAN J. MOSHER and P. TIMOTHY POLLAK

College of Pharmacy and Department of Medicine, Faculties of Health Professions and Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 3J5

Abstract: Amlodipine is a long acting dihydropyridine calcium antagonist recently introduced for the treatment of angina and hypertension. In order to document its stability *in vitro* and to develop a pharmacokinetic model in rabbits, a new reversed-phase liquid chromatography (LC) assay with UV detection was developed. The method utilized a C_{18} column (250 × 4.6 mm i.d.) with a mobile phase composed of a mixture of methanol 0.04 M ammonium acetate–acetonitrile (38:38:24, v/v/v) containing 0.02% triethylamine (final pH 7.1). Under these conditions, the retention times of amlodipine and the internal standard desipramine were 10.6 and 12.9 min, respectively. Using 1 ml of plasma, sensitivity of the assay was 2.5 ng ml^{-1} at which the RSD was 11%. The standard curve was linear from 2.5 to 100 ng ml^{-1} (r^2 = 0.990), and the mean RSD at this concentration range was 6.8%. The pharmacokinetic model was developed in rabbits which provides results similar to those in dogs, but at less expense.

The assay was also applied to a stability study comparing amlodipine and nifedipine in pH 3 and pH 7 ammonium acetate buffers and in methanol. Amlodipine was considerably more stable than nifedipine under all conditions. Finally the assay was applied to a pharmacokinetic study in rabbits (n = 6) after a single 1 mg kg^{-1} intravenous dose. The mean half-life ($t_{½}$) of amlodipine was 6.5 h, the systemic clearance (CL) was 4.8 l h^{-1} kg^{-1} and the apparent volume of distribution at steady state (V_{dss}) was 30.2 l kg^{-1}. In conclusion, the LC assay is simple and sensitive, and should be applicable to pharmaceutical analysis, pharmacokinetic studies and therapeutic drug monitoring although the latter requires validation.

Keywords: *Amlodipine; calcium antagonists; reversed-phase HPLC; pharmacokinetics; angina; hypertension.*

Introduction

Amlodipine, R,S-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine (Fig. 1), is a new dihydropyridine calcium antagonist with a slow onset of vasodilatory action [1, 2]. Compared with nifedipine and other dihydropyridine calcium antagonists, amlodipine has a longer terminal half-life ($t_{½}$), higher oral bioavailability (F), and can be used with a once-a-day dosage schedule to control angina and hypertension [3, 4].

In order to study the pharmacokinetics and pharmacodynamics of amlodipine, a sensitive assay practical for routine use is required. Previously reported assays have included chemical derivatization of amlodipine followed by analysis with capillary gas chromatography (GC) and electron-capture detection [5]. Such an assay is capable of detecting less than 1 ng ml^{-1} of amlodipine in a 1 ml sample of plasma. However, the GC assay suffers from the risk of on-column oxidation of amlodipine due to high operating temperature [5, 6]. Liquid chromatography (LC) assays have also been described for amlodipine. The LC assay with amperometric detection [7] is as sensitive as the GC assay [5], but details of the LC assay utilizing fluorescence detection [8] have not been evaluated.

Experimental

Materials

Amlodipine was received as a gift from Drs D. Mehta and M. Jawadekar of Pfizer Central Research (Groton, CT, USA). Nifedipine was supplied by Miles Pharmaceuticals of Canada (Ontario, Canada). The internal standard desipramine was obtained from Merrell Dow Pharmaceuticals (Ontario, Canada). LC and extraction solvents were all of HPLC grade (BDH

*Part of the material in this manuscript was presented at the Fifth Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists, Las Vegas, Nevada, USA, November 1990.

† Author to whom correspondence should be addressed.

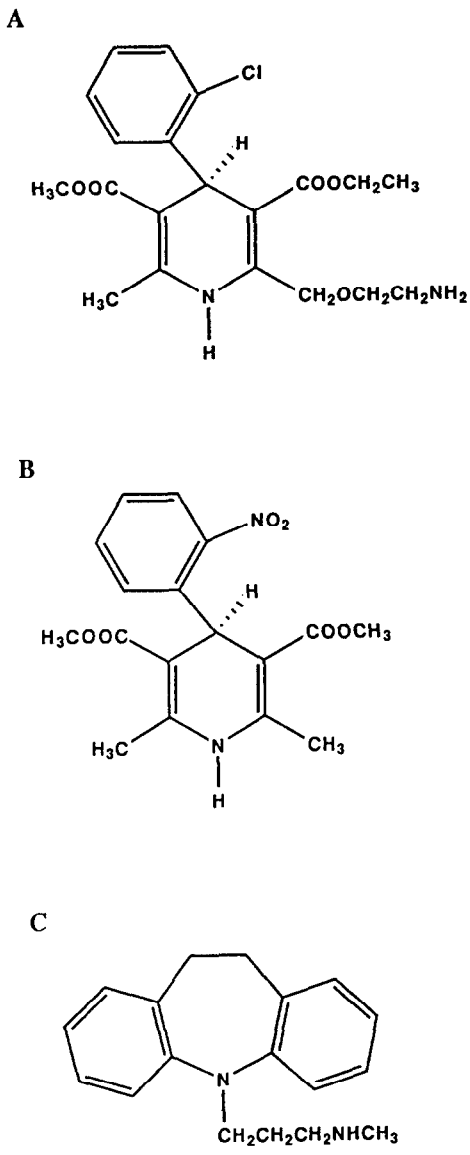

Figure 1
Chemical structures of (A) amlodipine, (B) nifedipine and (C) desipramine.

Chemicals, Nova Scotia, Canada). All other chemicals were of reagent grade (Fisher Scientific, Nova Scotia, Canada).

Instrumentation

The LC system from Beckman Instruments (Berkeley, CA, USA) consisted of a model 114 M solvent delivery system, a model 210A injection valve with a 50 μl loop, a model 163 variable wavelength UV detector, a model 427 integrator, an analytical Ultrasphere-ODS (250 × 4.6 mm i.d., 5 μm) and guard (45 × 4.6 mm i.d., 5 μm) columns. The mobile phase consisted of a mixture of methanol–0.04 M ammonium acetate–acetonitrile (38:38:24, v/v/v) containing 0.02% triethylamine, with the final pH adjusted to 7.1 using glacial acetic acid. The mobile phase was filtered and deaerated through a 0.45-μm membrane filter (Millipore, Ontario, Canada). The LC system was operated isocratically at ambient temperature with a flow rate of 1.2 ml min^{-1}. The detector wavelength was adjusted to 240 nm and sensitivity set at 0.04 AUFS to provide optimum response for amlodipine under the described conditions.

Standard solutions

Stock solutions of amlodipine and desipramine hydrochloride were prepared by dissolving 10 mg of the free base materials in 10 ml of methanol. Aliquots were subsequently diluted with methanol to yield 0.1 mg ml^{-1} stock solutions. All stock solutions were stored at 4°C in the dark, and were found to be stable for at least 1 month. A plasma standard at 100 ng ml^{-1} was prepared by spiking blank plasma obtained from drug-free rabbits with the stock solution of amlodipine. Subsequent serial dilution of this 100 ng ml^{-1} standard provided plasma standards of 50, 25, 10, 5 and 2.5 ng ml^{-1}. On the day of analysis, a working solution of the internal standard desipramine was prepared in 0.01 N hydrochloric acid (HCl) to a final concentration of 2 μg ml^{-1}.

Extraction procedure

To a 16 × 100 mm test tube with a PTFE-lined screw cap (Kimax Brand, Fisher Scientific, Canada), 1 ml of plasma sample or standard, 0.1 ml of the working internal standard solution (200 ng desipramine), and 0.2 ml of a 10% ammonium carbonate solution (final pH ~8.7) were added. The sample was gently mixed (Multi-tube vortexer, Canlab, New Brunswick, Canada), then extracted with 5 ml of methyl tert-butyl ether for 20 min (Vibrax VXR2, Terochem Laboratory, Ontario, Canada), and centrifuged (1720*g*) at 4°C for 10 min. The top organic layer was transferred to a 15-ml tapered centrifuged tube (Kimax Brand), and evaporated to dryness at 55°C (Thermolyn dri-bath, Fisher Scientific, Canada) under a gentle stream of nitrogen. The residue was reconstituted in 0.1 ml of 0.01 N HCl, and vortex washed with 2 ml of methyl tert-butyl ether for 1 min. The organic layer was discarded and the aqueous portion was dried at

68°C under high vacuum. The dried residue was kept at −20°C until analysis. It was reconstituted in 0.1 ml of mobile phase and an aliquot of this (20–40 μl) was injected into the HPLC. A standard curve of the assay was constructed from 2.5 to 100 ng ml^{-1} using unweighted linear regression analysis. Four separate extractions were carried out at each concentration.

Stability study

Standard solutions of amlodipine and nifedipine at 10 μg ml^{-1} were prepared in 0.05 M ammonium acetate buffers of pH 3 and 7, and in methanol. This concentration permitted the size of the aliquots removed for assay to be reduced to 5 μl. The solutions were placed in glass scintillation vials (Fisher Scientific, Nova Scotia, Canada), approximately 5 cm from a 15 W fluorescence light and aliquots were removed for assay after 2, 4, 6, 24, 30, 48 and 72 h. The 5-μl (50 ng) aliquots were assayed in quadruplicate for each solution before and at each period of exposure. Because it was only necessary to chromatograph the two pure substances for this portion of the experiment, amlodipine and nifedipine were assayed using a slightly modified mobile phase containing methanol–ammonium acetate (0.04 M)–acetonitrile (30:30:40) and 0.02% triethylamine with the final pH adjusted to 7.1 using glacial acetic acid. Under these conditions, the retention times of amlodipine and nifedipine were 5.1 and 4.6 min, respectively. Peak heights of amlodipine and nifedipine measured at the end of each time period were compared to those for the controls determined before exposure to light.

Pharmacokinetic study

The study protocol was approved by the Dalhousie University Committee on Laboratory Animals. Six male New Zealand white rabbits weighing from 3.3 to 4.0 kg were used in the study. On the day of the experiment, a 21G ¾″ butterfly catheter (Terumo™ Corp., Tokyo, Japan) was inserted into a central artery of each rabbit. Amlodipine (10 mg) was dissolved in a mixture of 5 ml of polyethylene glycol 200 (BDH Chem., Nova Scotia, Canada) and 10 ml of isotonic saline. This solution was injected over a 10-min period into the ear opposite the collection site such that each rabbit received 1 mg kg^{-1} of amlodipine. Blood samples (2 ml) were obtained via the catheter just before dosing and at 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0 and 8.0 h post-dose. The plasma was immediately separated by centrifugation at room temperature (4000*g*, 10 min) and stored at −20°C until analysis.

Pharmacokinetic parameters were calculated using a commercial curve fitting program (Rstrip®, MicroMath Scientific Software Inc., Salt Lake City, UT, USA). The data were fitted to a two-compartment model simulating bolus intravenous (i.v.) injection unless the data appeared more appropriate for a one-compartment model. Area under the plasma concentration–time curve from zero to infinity (AUC), area under the moment curve from zero to infinity (AUMC), and terminal plasma $t_{½}$ were obtained from the fitted data. Systemic clearance (CL) was calculated from the equation CL = Div/AUC, where Div was the intravenous dose. Mean residence time (MRT) was calculated by the expression MRT = AUMC/AUC, and apparent volume of distribution at steady state (V_{dss}) by V_{dss} = MRT × CL [9].

Results

Assay

Under the assay conditions described, amlodipine was clearly separated from the internal standard, desipramine, with retention times of 10.6 and 12.9 min, respectively. No endogenous plasma materials interfered with either peak (Fig. 2). Absolute recoveries of amlodipine by the extraction procedure were 78.5% at 100 ng ml^{-1} and 63.2% at 10 ng ml^{-1}. With the UV detector adjusted to 240 nm (λ_{max} for amlodipine) the limit of quantitation using 1 ml of plasma was about 2.5 ng ml^{-1} at which point the RSD was 11%. The mean RSD from 2.5 to 100 ng ml^{-1} was 6.8%. The standard curve was linear from 2.5 to 100 ng ml^{-1} (r^2 = 0.990) (Table 1). The accuracy of the assay was assessed by comparing nominal concentrations of amlodipine added to blank plasma with the observed values determined by the assay. Except at 50 ng ml^{-1}, the differences between the assay concentrations and theoretical values were <5%. The mean percentage difference, including the value at 50 ng ml^{-1}, was −2.8% (Table 2). The larger difference observed at 50 ng ml^{-1} could not be explained by a single outlier. When the accuracy at 50 ng ml^{-1} was assessed in other experiments carried out on other days, the mean assay concen-

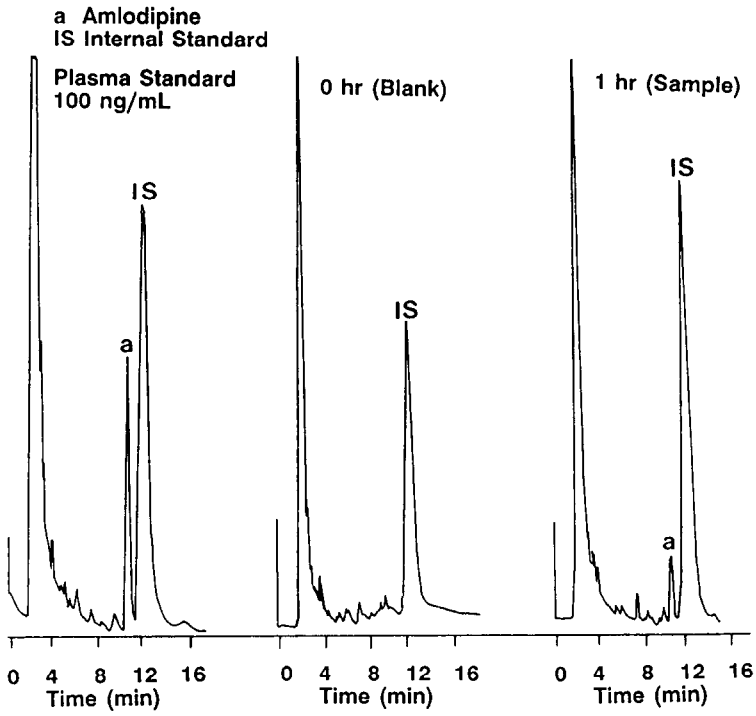

Figure 2
HPLC chromatograms of standard spiked sample, blank plasma and sample obtained from a rabbit 1 h after a single i.v. administration of amlodipine (detector sensitivity 0.04 AUFS).

Table 1
Standard curve data for the LC assay

Concentration (ng ml^{-1})	Peak height ratio*	RSD (%)
2.5	0.027 ± 0.003	11.1
5.0	0.050 ± 0.002	4.0
10.0	0.101 ± 0.010	9.9
25.0	0.274 ± 0.015	5.5
50.0	0.509 ± 0.027	5.3
100.0	1.270 ± 0.066	5.2

r^2 = 0.990
Slope = 0.013
Intercept = −0.032
Mean RSD = 6.8%

*Each value represents the within-day mean ±SD of four independent determinations.

tration was 47.8 ng ml^{-1} (n = 11), a difference of −4.4% from the theoretical value. The degree of deviation from the theoretical values did not show a trend which could be related to the concentration of the analyte (Table 2).

Stability of amlodipine in solution

Under the described experimental conditions, amlodipine was considerably more stable than nifedipine in all three *in vitro* systems. Compared to control (no light exposure), approximately 90% of amlodipine remained intact after 72 h of exposure to fluorescent light. In contrast, only 60% of nifedipine remained unchanged in the sol-

Table 2
Accuracy of the LC assay*

Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Difference from theoretical values (%)
10.0	10.0 ± 1.2%	0
25.0	24.3 ± 1.2%	−2.8
50.0	44.0 ± 0.3%	−12.0
100.0	103.5 ± 5.3%	+3.5
Mean % difference		−2.8

*Each value represents the within-day mean ±SD of four independent determinations.

utions after the first 6 h. Deterioration of nifedipine followed a biphasic pattern such that no major decrease in nifedipine concentrations occurred after 4 h (Fig. 3A–C). Stability of amlodipine and nifedipine did not appear to be affected by the pH of the buffer, or by the nature of the solvent (aqueous vs methanol) since the deterioration profiles were similar in all these solutions.

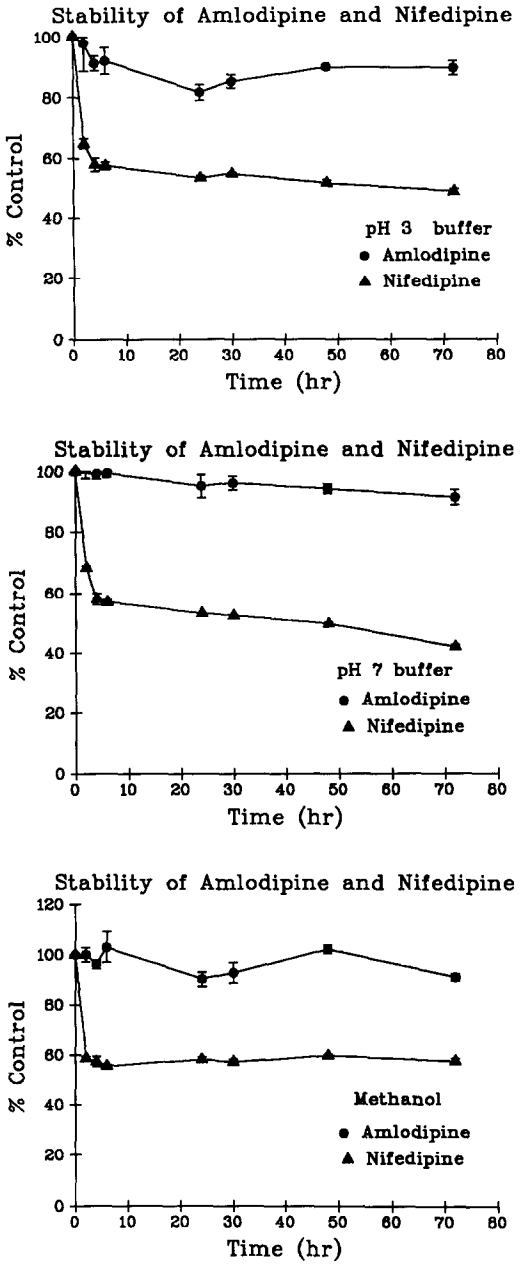

Figure 3
Stability of amlodipine and nifedipine: (A) ammonium acetate buffer pH 3, (B) ammonium acetate buffer pH 7, and (C) methanol. Each value represents the mean ±SD of four independent determinations.

Pharmacokinetic study

Following a single 1 mg kg^{-1} i.v. dose, plasma concentrations of amlodipine were best described by a two-compartment model, with a mean terminal $t_{½}$ of 6.5 h (Fig. 4). The mean CL and V_{dss} of amlodipine were 4.8 l h^{-1} kg^{-1} and 30.2 l kg^{-1}, respectively. There were noticeable intra-species variations in the disposition parameters of amlodipine among the six animals studied. Other selected pharmacokinetic parameters are listed in Table 3.

Discussion

During development of the assay, different methods of sample clean-up were evaluated. It was noted that methyl tert-butyl ether was effective in removing amlodipine and desipramine from plasma, but it also extracted endogenous plasma materials that interfered with the analysis. Attempts to back extract the analytes into different forms of acidic aqueous media were unsuccessful because the results varied considerably within the same experiment and between different working days. This problem was overcome by first evaporating the methyl tert-butyl ether to dryness. The

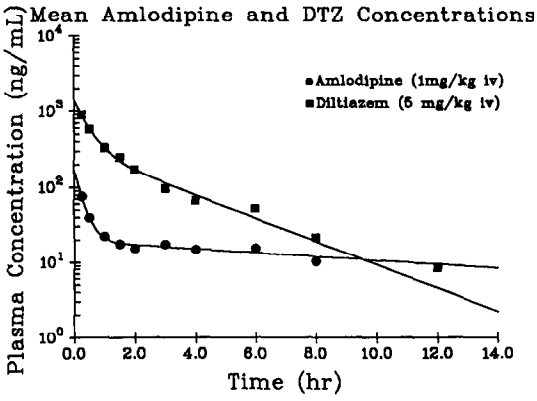

Figure 4
Mean plasma concentration–time profiles of amlodipine and diltiazem in rabbits following a single i.v. administration (n = 6 for each drug).

Table 3
Selected pharmacokinetic parameters of amlodipine in rabbits*

$t_{½}$ (h)	6.54 ± 4.34	(2.33–13.60)
AUC (ng-h ml^{-1})	257.2 ± 172.2	(150.2–562.9)
CL (l h^{-1} kg^{-1})	4.84 ± 1.84	(1.78–6.66)
V_{dss} (l kg^{-1})	30.2 ± 14.4	(10.8–51.3)
MRT (h)	8.01 ± 6.20	(1.62–17.9)

* Each value represents the mean ±SD of six rabbits and the range is in parenthesis.

residue was then reconstituted in 0.01 N HCl, which was subsequently washed with methyl tert-butyl ether to remove interfering materials. Acetic acid (1%) and acidic phosphate buffer systems were also tried as the aqueous media in this scheme, but the recovery was considerably lower than that of 0.01 N HCl. Although the described assay did not provide the sensitivity previously reported for capillary GC assay with electron capture detection [5] or the HPLC assay with amperometric detection [7], it does not require tedious derivatization or specialized detectors, making it readily amenable to routine laboratory use. The present LC assay has the advantage of eliminating the risk of thermal decomposition of amlodipine to pyridine products, and the assay reported here showed no interference from a panel of other therapeutic agents including propranolol, dipyridamole, nitroglycerin, verapamil, diltiazem, alprazolam, and chlorpromazine. These positive attributes suggest this assay would be useful for pharmacokinetic studies as well as therapeutic drug monitoring. However, the sensitivity requirement for use in clinical studies needs further evaluation and work is in progress to satisfy the criteria for applying this method to clinical studies.

Previous studies have shown that nifedipine and several other dihydropyridine calcium antagonists are unstable when exposed to UV light [10, 11]. The results of the present stability study also demonstrated rapid decomposition of nifedipine in methanolic, acidic or neutral aqueous solution. In comparison, amlodipine was much more stable than nifedipine under the same conditions despite similarities in structure.

The pharmacokinetic profile of amlodipine has been reported in humans, rats, dogs and mice [8]. The results of these studies showed that amlodipine has a large volume of distribution (range of 20–30 l kg^{-1}) and that CL was highest for rats (7.3 l h^{-1} kg^{-1}), followed by mice (1.7 l h^{-1} kg^{-1}), dogs (0.7 l h^{-1} kg^{-1}) and man (0.4 l h^{-1} kg^{-1}). Kinetic data in rabbits have not been reported previously. The calculated mean value of V_{dss} in rabbits reported here (30.2 l kg^{-1}) is comparable to those found in the other species. The CL (4.8 l h^{-1} kg^{-1}) observed in rabbits was considerably higher than those seen in man, dogs and mice, but somewhat lower than the values in rats. A similar pattern of species differences in the pharmacokinetic parameters of diltiazem has been reported previously [12]. These results suggest that the dog provides a model with pharmacokinetic parameters of amlodipine most comparable to man, but the rabbit offers advantages over the dog in terms of cost and size.

As noted in both animal and human studies, diltiazem, a benzothiazepine-derived calcium antagonist, has a shorter $t_{1/2}$ than amlodipine and therefore requires more frequent dosing in therapeutic situations [12]. The current rabbit studies further confirm the difference in the pharmacokinetic parameters of these two agents (Fig. 4). The mean $t_{1/2}$ and CL of diltiazem in rabbits after a single 5 mg kg^{-1} i.v. dose were 3.6 h and 3.8 l h^{-1} kg^{-1}, respectively [13]. The longer $t_{1/2}$ of amlodipine (6.5 h) compared to diltiazem was apparently not due to a lower CL, since the observed value for amlodipine (4.8 l h^{-1} kg^{-1}) was higher. Rather, the difference in $t_{1/2}$ appeared to be due to higher volume of distribution (amlodipine 30.2 l kg^{-1} vs diltiazem 6.8 l kg^{-1}). These results suggest that animal models such as the rabbit are useful for discriminating pharmacokinetic differences between amlodipine and diltiazem even though they do not precisely predict the values of pharmacokinetic parameters in humans. Whether or not a rabbit model is also capable of demonstrating pharmacokinetic differences amongst other calcium antagonists remains to be investigated. In view of the calculated $t_{1/2}$ of 6.5 h, it is apparent that the 8-h sampling schedule used in this study was not long enough to accurately characterize the terminal elimination of amlodipine. Thus the pharmacokinetic parameters reported here for amlodipine must be interpreted with caution. Further studies with an appropriately long sample collection period will be required to calculate the terminal elimination constant in rabbits.

Acknowledgements — The authors thank Drs D. Mehta, J. Flax and M. Jawadekar of Pfizer Central Research (Groton, CT, USA) and M.J. Humphrey (Pfizer Central Research, Sandwich, Kent, UK) for their review and support of this research.

References

[1] J.E. Arrowsmith, S.F. Campbell, P.E. Cross, J.K. Stubbs, R.A. Burges, D.G. Gardiner and K.J. Blackburn, *J. Med. Chem.* **29**, 1696–1702 (1986).

[2] R.A. Burges, D.G. Gardiner, M. Gwilt *et al.*, *J. Cardiovas. Pharmac.* **9**, 110–119 (1986).

[3] E.R. Bühler, *J. Cardiovas. Pharmac.* **12** (Suppl. 7), S1–S2 (1988).
[4] D.R. Abernethy, *Am. Heart J.* **118**, 1100–1103 (1989).
[5] A.P. Beresford, P.V. MaCrae, D.A. Stopher and B.A. Wood, *J. Chromat. (Biomed. Appl.)* **420**, 178–183 (1987).
[6] M. Abnoff, *J. Pharm. Biomed. Anal.* **2**, 519–526 (1984).
[7] K. Shimooka, Y. Sawada and H. Tatematsu, *J. Pharm. Biomed. Anal.* **7**, 1267–1272 (1989).
[8] D.A. Stopher, A.P. Beresford, P.V. MaCrae and M.J. Humphrey, *J. Cardiovas. Pharmac.* **12** (Suppl. 7), S55–S59 (1988).
[9] M. Gibaldi and D. Perrier, *Pharmacokinetics*, 2nd edn. Marcel Dekker, New York (1982).
[10] F.A. Tucker, P.S.B. Minty and G.A. MacGregor, *J. Chromat.* **342**, 193–198 (1985).
[11] P.O. Greiner, D. Angignard and J. Cahn, *J. Pharm. Sci.* **77**, 387–389 (1988).
[12] P.K.F. Yeung, S.J. Mosher, M.A. Quilliam and T.J. Montague, *Drug Metab. Disp.* **18**, 1055–1059 (1990).
[13] P.K.F. Yeung, S.J. Mosher and P.T. Pollak, *Eur. J. Drug Pharmac.* **16**, 69–74 (1991).

[Received for review 17 April 1991; revised manuscript received 19 July 1991]