

Enzyme Linked Immunosorbent Assay for Determination of Amlodipine in Plasma

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Amlodipine is a calcium channel antagonist of the dihydropyridine group. It is effective for treating hypertension, chronic stable angina, and vasospastic angina. However, it is difficult clinically to pinpoint the maximum dosage for antihypertensive activity of the drug without having parallel data on the plasma drug concentrations. The methods for assaying amlodipine are either gas chromatography with electron capture detector or liquid chromatography coupled with tandem mass spectrometry (or with an electrochemical detector), which needs tedious derivatization, and is expensive and time consuming. Therefore, in this study we developed an enzyme immunoassay for determining amlodipine in plasma. Anti-amlodipine antibodies were produced following immu-

nization of bovine serum albumin-amlodipine conjugate. These specific antibodies were used in a competitive biotin-avidin-based enzyme-linked immunosorbent assay to measure amlodipine in plasma. Biotin was linked to the antibodies in order to enhance the sensitivity of the assay. The assay was specific for the free form of amlodipine with a detection limit of 0.1 ng/ml and the intra- and interassay coefficient of variation ranged from 1.6–10.2%. This immunoassay provides a sensitive, reliable, rapid, and accurate method for determination of amlodipine in plasma, which can be used in therapeutic drug monitoring pharmacokinetic studies and pharmaceutical analysis. *J. Clin. Lab. Anal.* 15:47–53, 2001. © 2001 Wiley-Liss, Inc.

Key words: ELISA; amlodipine; anti-amlodipine antibodies; nifedipine

INTRODUCTION

Amlodipine (3-ethyl 5-methyl -2-[2-aminoethoxy]methoxy]-4-(o-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate monobenzenesulfonate) is a calcium channel antagonist of the dihydropyridine group. It is effective for treating hypertension, chronic stable angina, and vasospastic angina. It acts by exhibiting potent peripheral and coronary artery vasodilation, high vascular selectivity, and little or no effect on cardiac conduction or contractility (1,2).

The recommended dosages for amlodipine for use as an antihypertensive drug are based on clinical trials in which doses are increased until the desired effect is achieved or until unacceptable side effects, such as peripheral edema, are observed (3). Furthermore, it is difficult clinically to pinpoint the maximum dosage for antihypertensive activity of the drug without having parallel data on the plasma drug concentrations. This is noted in the literature: several studies arrived at different conclusions for how best to achieve satisfactory blood pressure control in hypertensive patients when treated with amlodipine or other antihypertensive agents (4–7).

The available methods for assaying amlodipine in biological fluids are gas chromatography with electron capture detector (GCECD) (8,9), high-performance thin-layer chromatography (HPTLC) (10), liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) (11), or with electrochemical detector (HPLC-EC) (12). But the GCECD, LC-MS-MS, and HPLC-EC methods require tedious derivatization, the GCECD method suffers from the risk of on-column oxidation of amlodipine due to high operating temperatures, and the HPTLC is not sensitive enough. All in all, these methods are expensive and time consuming (8–12). In this study, therefore, we developed a simple, rapid, sensitive and accurate enzyme linked immunosorbent assay (ELISA) method for assaying amlodipine in biological fluids. This method was applied to a pharmacokinetic study of amlodipine

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in humans, in which the measurable range was 0.1–40 ng/ml using 50 μ l of plasma.

MATERIALS AND METHODS

The following chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO): bovine serum albumin (BSA), ovalbumin (OVA), IgG goat antirabbit alkaline phosphatase conjugate, extraAvidin alkaline phosphatase conjugate, glutaraldehyde 25% (v/v), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC), p-nitrophenyl phosphate, Tween-20, glycine, sodium azide, diethanolamine, avidin, biotinamidocaproate-N-hydroxy-sulfosuccinimide ester, G25 Sephadex, pronase, 10 mM 4'-hydroxyazobenzene-2- carboxylic acid, Freund's complete adjuvant, and Freund's incomplete adjuvant. Tris buffer, sodium chloride, and ammonium sulfate were purchased from Acros Organics (Springfield, NJ). Hitrap Protein A column was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden), and protein microassay was purchased from BioRad Laboratories (Richmond, CA). Microtiter 96-well flat-bottom plates were purchased from Greiner, Labor-technik (Frickenhause, Germany). The ELISA Wellsan reader and Wellwash were obtained from Denley Co. (Billinghurst, UK). Centricon-30 concentrators were obtained from Amicon Inc. (Beverly, MA).

Preparation of BSA-Amlodipine Immunogen

Amlodipine was covalently linked to BSA using a homobifunctional cross-linker glutaraldehyde (13). Amlodipine (38 mg) was dissolved in 1 ml methanol. From this amlodipine solution, a volume of 0.32 ml was mixed with 0.68 ml of 0.4 M phosphate buffer, pH 7.5 and added to BSA (23 mg) solution dissolved in 1 ml of 0.4 M sodium phosphate buffer, pH 7.5. To the mixture were added 8.6 mg of glutaraldehyde in 34 μ l of 0.4 M sodium phosphate buffer, all of which was incubated, with mixing, for 30 minutes. Glycine (64.5 mg) was dissolved in 0.5 ml dH₂O, added to the mixture, and left to incubate for another 30 minutes. The BSA-amlodipine conjugate was purified by gel chromatography (2 \times 30 cm) of Sephadex-G25, equilibrated in PBS, pH 7.3. The eluted fractions were monitored for protein at 280 nm. Protein fractions were pooled and protein concentration was measured.

Immunization

White New Zealand rabbits were injected subcutaneously (s.c.), adhering to the National Institutes of Health's publication "Principles of Laboratory Animal Care" (#85-23, revised 1985), with 200 μ g of BSA-amlodipine conjugate in 3.5 ml PBS, pH 7.3 mixed with 1.5 ml Freund's complete adjuvant. The suspension was injected s.c. at ten different sites on the animals back. Booster injections were performed after one and two months using the same mixture but with Freund's

incomplete adjuvant. Rabbits were bled 12 days after the last booster, blood was left to clot, was centrifuged, and serum was aliquoted and stored at -20°C.

Antibody Purification

The IgG fraction of antisera was prepared by ammonium sulfate precipitation followed by affinity chromatography protein A column. Briefly, rabbit sera were diluted 1:2 with normal saline and then saturated ammonium sulfate, pH 7.2, was added to make a final concentration of ammonium sulfate 45% (v/v). The mixture was stirred for 30 minutes at room temperature and centrifuged at 1000g for 15 minutes. The precipitate was washed again with 45% ammonium sulfate, and recentrifuged. The precipitate was then dissolved with PBS, pH 7.3, and centrifuged again to remove any insoluble material. The supernatant was precipitated again with 40% ammonium sulfate, centrifuged, and redissolved with 2-4 ml of PBS, pH 7.3. The final solution was dialyzed (2L) with five changes of PBS, aliquoted and stored at -20°C.

Ammonium sulfate-precipitated gamma fractions were further purified by protein A column. Briefly, the column was washed with a binding buffer, 0.02 M phosphate buffer, pH 8.5, containing 1M NaCl, and the gamma fractions were loaded on the column by pumping 50 ml of 2 mg/ml protein diluted with binding buffer at 1 ml/min. The column was then washed with 20 ml of binding buffer. IgG fractions (2 ml) were eluted using 0.1M citrate buffer, pH 4.0, immediately neutralized, and monitored at 280 nm. The protein-containing fractions were pooled and dialyzed against five changes of PBS.

Biotinylation of Anti-Amlodipine Antibody

The IgG solution was concentrated to ~ 10 mg/ml using centricon-30 concentrators in 0.1 M phosphate buffer, pH 7.2. Added directly to 3 ml of anti-amlodipine IgG solution were 1.3 mg of biotinamidocaproate-N-hydroxy-sulfosuccinimide ester, which were then mixed for 30 minutes at room temperature, and purified by gel filtration on a G25 Sephadex column (14). The biotin/Ab ratio was determined using avidin and 10 mM 4'-hydroxyazobenzene-2- carboxylic acid, HABA-assay (15). The absorption of the avidin-HABA complex at 500 nm decreases proportionally with increased concentration of biotin in the solution, where biotin binds to avidin displacing HABA dye. The ratio of biotin to Ab was approximately 4:1.

Preparation of Ovalbumin-Amlodipine Conjugate

Ova-amlodipine conjugate was prepared for use in the ELISA for coating amlodipine to the 96-well plates. A different cross-linker was used for the coating material to avoid interference with the sensitivity of the assay by antibodies that may be raised to the cross-linker, glutaraldehyde. Ovalbumin (23 mg) was dissolved in 1.5 ml dH₂O and mixed with 9.8 mg of amlodipine in 82 μ l of methanol. EDAC (6 mg) in

227 μ l of dH₂O was added to the mixture, and incubated at room temperature overnight with gentle mixing. The conjugate was purified on a G25 Sephadex column, and the eluted fractions in PBS, pH 7.3, were monitored for protein at 280 nm. The protein fractions were pooled, aliquoted, and kept frozen at -20°C .

ELISA Amlodipine Assay

Flat-bottom 96-well microtiter plates were coated with 0.1 ml of 3.2 $\mu\text{g/ml}$ of Ova-amlodipine in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6, and left overnight at room temperature. Wells were washed three times with 25 mM Tris-HCl buffer, pH 7.3, containing 0.05% Tween-20, 0.15 M NaCl, and 0.02% sodium azide, blocked with the same washing buffer for 30 minutes, and washed twice (16,17). For anti-amlodipine Ab titration, serial dilutions of antibody (with or without biotin) in assay buffer (50 mM Tris-HCl buffer, containing 1% BSA, pH = 7.3, 0.05% Tween-20, 0.15 M NaCl and 0.02% sodium azide) were added to the wells in 0.1 ml/well and left incubating at 22°C for 2 h with orbital shaking. The wells were washed 3 times, and diluted goat antirabbit or extraAvidin alkaline phosphatase conjugates (in assay buffer) were added in 0.1 ml/well and left incubating for 1 h at 22°C with orbital shaking. The wells were washed 4 times, and 0.1 ml/well of 1 mg/ml of p-nitrophenyl phosphate in 10% diethanolamine, pH 9.8, containing 1 mM MgCl₂ and 0.02% sodium azide was added. After 30 minutes, color development was stopped by the addition of 2 M NaOH (0.1 ml/well), and absorbance was read at 405 nm and 600 nm differential filter.

For amlodipine assay, 1 mg/ml of amlodipine in methanol was prepared and then diluted in assay buffer or pooled heparinized plasma. Standards were added in 50- $\mu\text{l/well}$ units followed by 50 $\mu\text{l/well}$ of anti-amlodipine Ab (with or without biotin) to coated and blocked wells, as described previously. The rest of the assay was performed as described. Absorbance values were transformed to percent binding and then to logit binding to construct a straight line on a semi-log graph paper.

Determination of Amlodipine Levels in Plasma Following a Single Dose

This study was conducted at Al-Mowasah Hospital (Amman, Jordan) and was approved by the hospital's ethical committee. The study was performed in accordance with the relevant articles of the Declaration of Helsinki (1964) as revised in Tokyo (1975), Venice (1983), Hong Kong (1989), and Somerset West, RSA (1996). Each volunteer had agreed to participate in the study and signed an informed consent prior to the initiation of the study.

Twenty-four healthy fasting male volunteers with mean age and weight of 27.6 ± 5.8 years and 76.0 ± 8.6 Kg, respectively, received a 5-mg single oral dose of amlodipine tablet (Norvasc, Pfizer, Lot No. 901-05603). Blood samples were

collected in heparinized tubes at 0, 1, 2, 3, 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11, 12, 24, 48, 72, 96, 120, and 144 h after dosing. Plasma was separated, stored at -20°C and assayed later using the aforementioned method.

RESULTS

Production and Specificity of Anti-Amlodipine Ab

BSA-amlodipine conjugate proved to be highly immunogenic and the antibodies produced were specific to amlodipine. The ammonium sulfate gamma fractions and protein A purified IgG were used in testing the specificity of the antibody and in the amlodipine assay. The specificity of the anti-amlodipine antibody was found to be directed towards A and B sites (Fig. 1). This was determined by testing the anti-amlodipine Ab cross-reactivity with nicotinic acid, 2-chloro-5-nitro-benzoic acid, and nifedipine (Fig. 2). Nicotinic acid and 2-chloro-5-nitro-benzoic acid showed no cross-reactivity, the inhibitory concentration at 50% (IC 50%) was not reached even at 10^{-3} M. Nifedipine, which

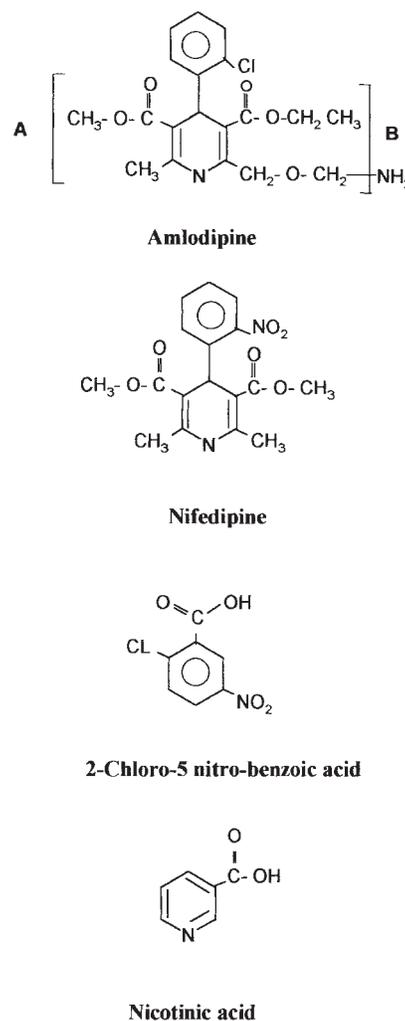


Fig. 1. Structures of amlodipine, nifedipine, 2-chloro-5-nitro-benzoic acid, and nicotinic acid. A and B show the anti-amlodipine Ab cross-reactions.

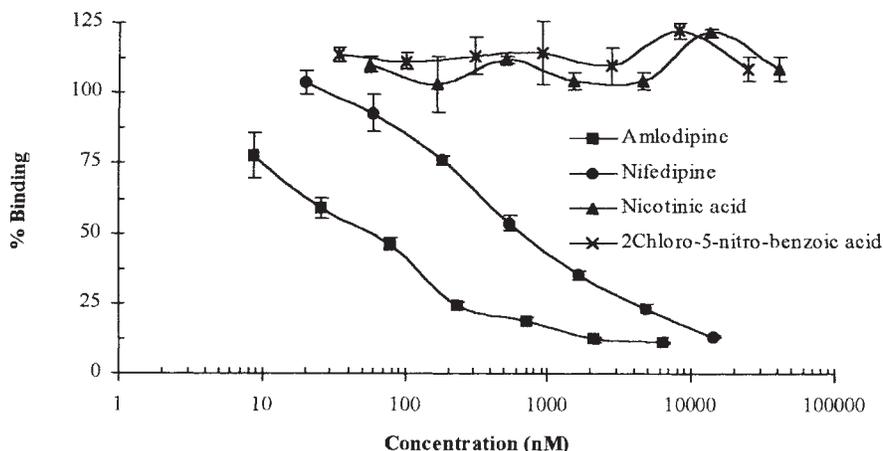


Fig. 2. Competitive ELISA between OVA-amlopidine-coated wells and amlodipine, nifedipine, 2-chloro-5-nitro-benzoic acid, or nicotinic acid with

anti-amlopidine Ab (ammonium sulfate gamma fraction). The points in the figure represent the mean of triplicate readings \pm SD.

is structurally and functionally related to amlodipine, however, showed IC₅₀% of 600–650 nM (Fig. 2).

Competitive ELISA for Amlodipine in Plasma

The anti-amlopidine Ab from the ammonium sulfate or protein A did not show the desired sensitivity and competition at the range of 0.5–10 ng/ml (Fig. 2). Thus the biotin-avidin system was used because avidin has 4 binding sites to biotin and, therefore, sensitivity should be enhanced. The anti-amlopidine-biotin Ab retained its immunoreactivity (80%) when compared to the parent antibody (Fig. 3) and was used in the competitive assay for amlodipine followed by extraAvidin alkaline phosphatase conjugate. In the competitive ELISA, the biotin-avidin system gave better sensitivity and competition at the desired range of concentration (Fig. 4). The standard curves of the assay exhibited good linearity ($r^2 = 0.989$ – 0.995), when logit-transformed data was plotted versus concentration of amlodipine on semilog paper (Fig. 5).

The performance of the assay was determined by its accuracy, precision, and sensitivity following intra- and interassay validations (Table 1). The intra- and interassay accuracy (% recovery) ranged from 83–115 and 92–112%, respectively, over 0.5–40 ng/ml range of amlodipine in human plasma. The intra- and interassay precision (% CV) ranged from 1.2–10.6 and 2.6–10.2%, respectively, over 0.5–40 ng/ml range of amlodipine in human plasma. The sensitivity or the lower limit of detection was defined by the concentration of amlodipine equivalent to the mean absorbance minus 2SD, $X - 2SD$, in 8 replicates, and was assessed to be 0.1 ng/ml, which corresponds to 90% binding.

Determination of Amlodipine Levels in Plasma Following a Single Dose

The mean amlodipine concentrations (\pm SE) versus time following a single dose of 5 mg of amlodipine is shown in Fig. 6. The range of individual maximum plasma concentra-

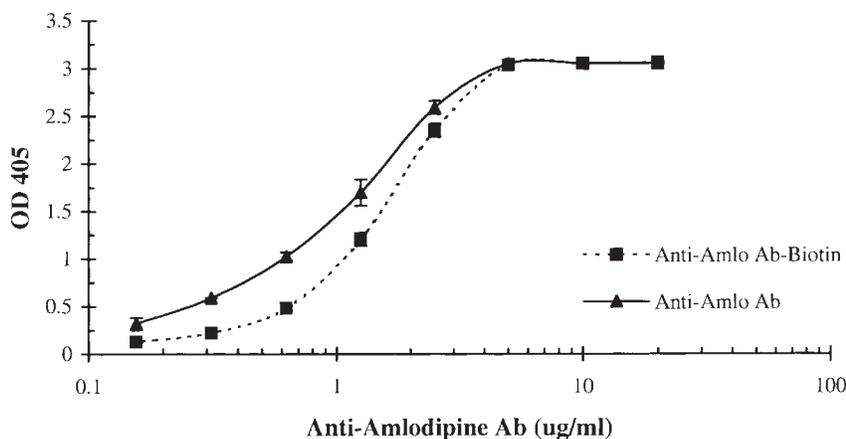


Fig. 3. Titration curve of anti-amlopidine Ab (protein-A purified) and anti-amlopidine Ab-biotin conjugate. The points in the figure represent the mean of tetraplicate readings \pm SD.

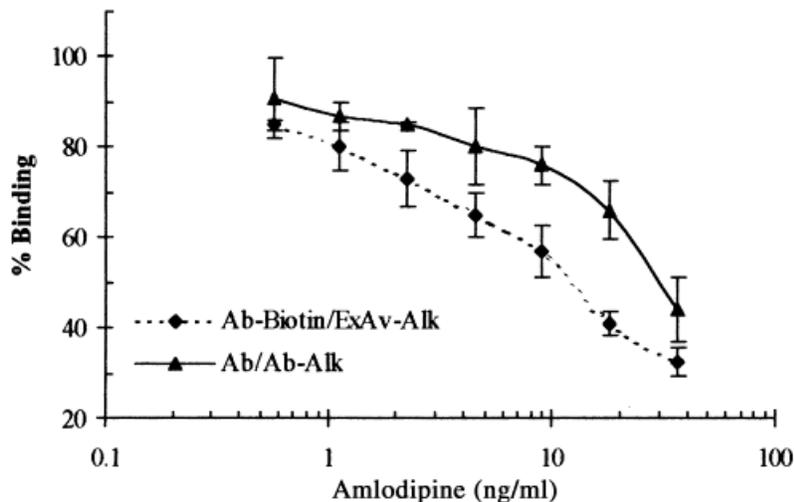


Fig. 4. Competitive ELISA between OVA-amlodipine-coated wells and amlodipine in plasma with anti-amlodipine Ab using either the protein-A purified Ab or the anti-amlodipine Ab-B4 conjugate. The antibody alkaline

phosphatase and extrAvidin alkaline phosphatase conjugates were used with protein-A purified Ab and Ab-B4, respectively. The points in the figure represent the mean of tetraplicate readings \pm SD.

tions was 1.49–5.74 ng/ml with a mean value ($C_{max} \pm SD$) of 3.30 ± 1.30 ng/ml and the time required to reach these maximum plasma concentrations ($t_{max} \pm SD$) was 7.60 ± 1.58 h. The area under the plasma concentration-time profile ($AUC \pm SD$) was 83.6 ± 38.0 ng.h/ml.

DISCUSSION

The present study shows a new method for measuring amlodipine in biological fluids such as plasma or serum, using an enzyme immunoassay. A specific anti-amlodipine Ab was produced following immunizing rabbits with BSA-

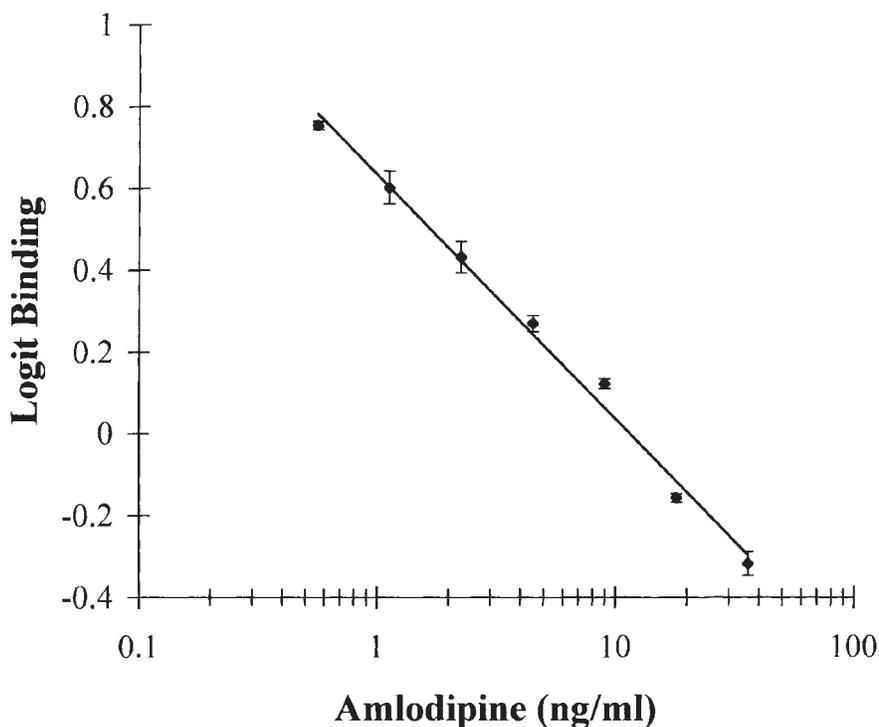


Fig. 5. A typical calibration curve of amlodipine in plasma with $r^2 = 0.9917$. The points in the figure represent the mean of tetraplicate readings \pm SD.

TABLE 1. Performance of the ELISA method for assaying amlodipine in plasma

Theoretical Amlodipine Conc. (ng/ml)	Measured Amlodipine Conc. (ng/ml)					
	Intra-assay (n = 8)			Inter-assay (n = 16)		
	Conc.	% Recovery	% CV	Conc.	% Recovery	% CV
0.5	0.42	83.3	1.56	0.48	96	2.6
1.0	1.15	114.9	1.87	1.12	111.7	2.6
2.5	2.39	95.4	4.02	2.27	96.7	6.7
5.0	4.29	85.8	4.85	4.6	92	9.5
10	11.5	115.3	5.74	10.4	104	9.2
40	38.2	95.5	5.18	39.2	98.2	10.2

amlodipine conjugate. The antibody's binding sites were determined to be specific to amlodipine but to cross-react with a similar structure as seen with nifedipine, a closely related calcium channel blocker. Nifedipine showed approximately 10% cross-reactivity in our assay (Fig. 2).

The anti-amlodipine Ab developed here binds only the free form of amlodipine as shown with the amlodipine plasma concentrations following a 5-mg dose. The maximum plasma concentration was assayed to be 3.3 ng/ml, which is similar to published data performed by GCECD (8,18). T_{max} and AUC were also within the range of the published data (18–20).

Amlodipine has a pKa of 8.7 (19) and is highly bound to proteins (up to 93%) and 7% only present as the free form (2,10,19). The presence of the basic side chain at the 2-position of the dihydropyridine ring renders amlodipine to be ionized (> 90%) at physiological pH and at this site where

amlodipine binds electrostatically to proteins in plasma (20). The immunoconjugate presented in this study was covalently made with a spacer between the amino group of amlodipine and the carrier protein, and therefore the antibody was found to detect only the free form of amlodipine. Because our anti-amlodipine Ab did not detect the bound fraction of amlodipine in plasma, it could be postulated that proteins in plasma fold over amlodipine and thus the antibody was unable to bind the bound form of amlodipine. However, total amlodipine could be measured by treating plasma with pepsin to induce proteolysis, then amlodipine is extracted and assayed by ELISA (21).

The application of such a method is establishing the recommended dosages for amlodipine as an antihypertensive drug in order to achieve the desired effect without unacceptable side effects. Furthermore, it could be used to determine the clinical significance of certain food–amlodipine interaction or with any other drug (22). In addition, it can facilitate studying the interactions between amlodipine and cytokines, such as interleukin 1 (IL-1), IL-6, tumor necrosis factor- α , and nitric oxide (23–25).

The present study describes an immunoassay for determining amlodipine in plasma. This immunoassay determines the free form of amlodipine, and the pharmacokinetic parameters obtained by this method were similar to the data in the literature. However, the method described here is not only simpler and faster than the available methods for assaying amlodipine but also is sensitive (0.1 ng/ml) and can be used in clinical laboratories for therapeutic drug monitoring of amlodipine.

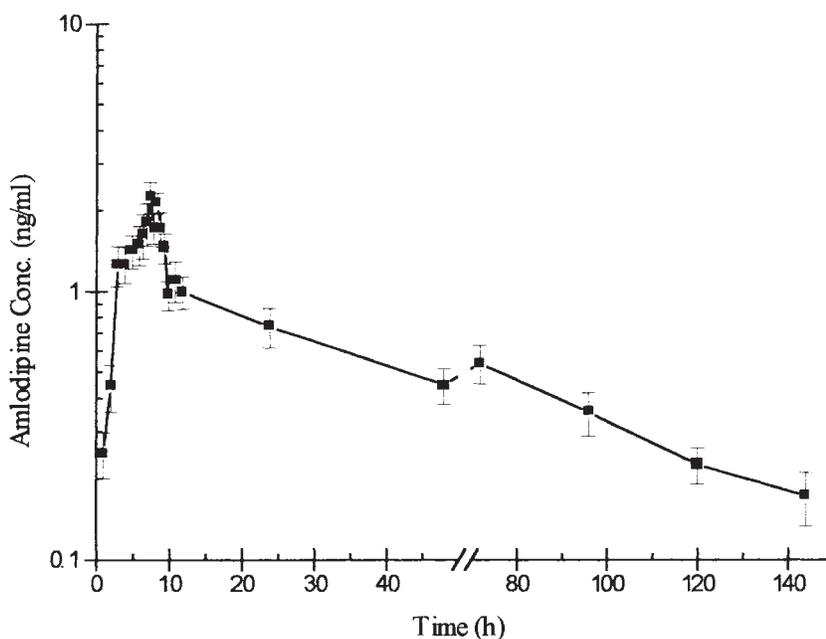


Fig. 6. Mean (\pm SE) amlodipine levels in plasma following a 5-mg oral dose of (Norvasc, in 24 healthy male volunteers.

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