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Enantioselective gas chromatographic assay with electron-capture detection for amlodipine in biological samples

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Abstract

A sensitive enantioselective gas chromatographic assay has been developed for amlodipine, 2-[(2-aminoethoxy)-methyl]-4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-1,4-dihydropyridine, a calcium channel blocking therapeutic agent. The assay involves conversion of the (+)-(*R*)- and (−)-(*S*)-enantiomers of amlodipine into their acyl derivatives with the chiral reagent (+)-(*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (Mosher's reagent). Peak separation after chromatography of the diastereomers was larger than 85%, and the lower limit of detection in blood plasma was 0.02 ng/ml for each enantiomer. The method has been used for the measurement of amlodipine enantiomers in human, rat and dog plasma, and in various organs of the rat.

1. Introduction

Amlodipine, a dihydropyridine derivative with calcium antagonistic activity [1], is clinically used in the treatment of hypertension and angina [2]. In man, it has the highest oral bioavailability and the longest half-life of elimination among the drugs of its class [3]. Like most other calcium channel blockers of the dihydropyridine type amlodipine is therapeutically used as a racemate. The pharmacological potency of the (−)-(*S*)-enantiomer of amlodipine has been found to be *ca.* 1000 times higher than that of the (+)-(*R*)-enantiomer [4,5], which implies a different pharmacokinetic behaviour of both enantiomers.

A sensitive gas chromatographic method for the determination of amlodipine in plasma has been published [6], which, however, does not permit the enantioselective quantification of the drug, a demand which is met by the method presented here. The method is based on chiral derivatization combined with high-resolution GC, and makes use of the enhanced electron-capturing properties of the derivatives.

2. Experimental

2.1. Materials

Amlodipine (racemate) besylate (UK, 48,340), (+)-*R*-amlodipine (UK 61,031), (−)-(*S*)-amlodipine (UK 63,929) and the internal standard UK 52829-42 (Fig. 1) were obtained from Pfizer Central Research (Sandwich, UK). 4-Dimethylaminopyridine, (+)-(*S*)-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPAC), boric acid and *tert.*-butyl methyl ether (TBME)

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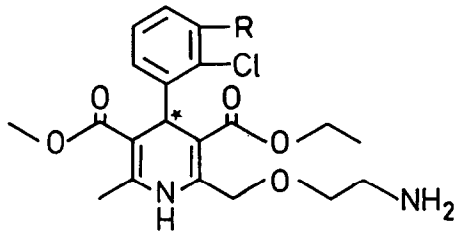

Fig. 1. Molecular structures of amlodipine (R = H) and the internal standard, UK 58829-42 (R = Cl), showing the asymmetric carbon atom (asterisk).

were from Fluka (Neu-Ulm, Germany). XAD type-2 adsorbent resin was from Serva (Heidelberg, Germany), Carbopack C 0.8% THEED 80/100 from ICT (Frankfurt, Germany), Sodium hydroxide solution (1 *M*), hydrochloric acid (0.1 *M*), citric acid monohydrate, potassium carbonate, dichloromethane, methanol and ethanol were purchased from Merck (Darmstadt, Germany). All solvents were of analytical grade except TBME, which was HPLC grade.

2.2. Preparation of solutions

Stock solutions of racemic amlodipine besylate in ethanol (100 μg/ml) and of the internal standard (UK 52829-42) in methanol (20 μg/ml) were stored at 4°C. These solutions were stable for several months. Appropriate dilutions were made immediately before use. Stock solutions of 200 μl MTPAC in 50 ml dichloromethane and of 10 mg 4-dimethylaminopyridine in 50 ml dichloromethane were prepared. Prior to use these solutions were mixed in a volume ratio of 1:1.

2.3. Instrumentation

A Dani Model 6500 gas chromatograph equipped with a ^{63}Ni electron-capture detector, a programmed temperature vaporizer (PTV), and an ALS 3940 autosampler (Dani, Monza, Italy) was used. The chromatographic signal was recorded and processed by a PE Nelson Analytical 2100 System (PE Nelson, Cupertino, CA, USA). Separations were carried out on a fused-silica capillary column, 30 m × 0.32 mm I.D., DB 1 bonded phase with 0.10-μm film thickness (J & W Scientific, Rancho Cordova, CA, USA). An automatically regenerable filter Type G 400 (Fehr, Hassloch, Germany) was used for purification of the carrier gas.

2.4. Gas chromatographic conditions

The initial column oven temperature of 65°C was held for 0.5 min and thereafter increased at a rate of 20°C/min to a final temperature of 290°C, which was maintained for 25 min. Initially the injector was operated in the split mode at 72°C (split ratio 1/50, septum purge 5 ml/min); 0.1 min after the injection the split valve was closed and the injector heated with a rate of 1300°C/min up to 290°C. After 1.7 min, *i.e.* the time required for complete transfer of the sample vapors into the column, the split valve was opened. The detector temperature was 310°C. The flow-rate of the carrier gas (hydrogen) was 1.5 ml/min and the flow-rate of the make up gas (nitrogen) was 40 ml/min.

2.5. Sample preparation

To 1 ml of plasma in a 15-ml glass tube 10 μl of the internal standard solution and 2 ml borate buffer (0.2 *M*) were added, and the sample was vortex-mixed for a few seconds. The mixture was extracted with 6 ml of *tert.*-butyl methyl ether for 40 min on a rotary mixer. The phases were separated by centrifugation at 1500 *g* for 4 min. The organic layer was transferred to another tube and extracted with 1 ml of 0.1 *M* citric acid for 30 min on the rotary mixer. The organic layer was discarded, and 2 ml of 1 *M* potassium carbonate solution were added to the aqueous extract, which was vortex-mixed for a few seconds. Then amlodipine and the standard were re-extracted into 6 ml of *tert.*-butyl methyl ether, as described above. A 100-μl volume of the MTPAC reagent solution was added to the organic phase, which was then mixed by rotation for 20 min and allowed to stand for 20 min at room temperature. A 1-ml volume of 1 *M* potassium carbonate was added to remove excess reagents, and the samples were mixed by rotation and centrifuged as above for a further 45 min. The organic layer was then evaporated to

dryness at 40°C under nitrogen. The residue was taken up in 50–200 μl ethyl acetate and aliquots of 0.3–1 μl were analyzed by GC–ECD.

For the extraction of amlodipine from tissue (brain, heart, lung, muscle, liver and kidney of the rat), 10–100 mg of the homogenized sample were vortex-mixed with 1 ml of 0.2 *M* borate buffer and the internal standard was added. The contents were extracted with 6 ml dichloromethane for 40 min on a rotary mixer and then centrifuged at 1500 *g* for 10 min. The aqueous phase was aspirated and the organic layer was transferred to a second tube containing 200 mg XAD-2. The mixture was shaken for 10 min. After centrifugation (1500 *g*, 4 min) the solution was again treated with an adsorbent (50 mg Carbopack C, 0.8% THEED) using the same procedure as for XAD-2. The organic layer was separated and the subsequent steps of extraction and back-extraction were carried out as described above for plasma.

2.6. Automated sample preparation

Since back extraction, derivatization and concentration of extracts are necessary but tedious and time consuming steps in the analysis for plasma samples, the procedure was adapted for performance by a robot. A Zymate II robot (Zymark, Hopkinton, MA, USA) was used, which consisted of the following components (number of modules in brackets): dual-function hand (2), liquid-transfer hand (1), power and event controller (2), rotating type shaker (1), centrifuge (1), evaporator (1), screw capper (1), crimp capper (1), vortex shaker (1), dispensary station (2), pneumatic dosing station (1), as well as various sample racks. All of the above-described steps of the assay, with the exception of measuring of the plasma volume, were performed by the laboratory robot. The last step carried out by the robot is the transfer of the derivatized plasma extracts into autosampler vials for gas chromatography. For serialization of the robotic sample processing the extraction and derivatization times (specified above) had to be altered slightly (±10%). This change has no effect on the accuracy of the method. The time required for the preparation of 50 samples was 30 h. This represents a total sample throughput of one sample per 36 min, which was similar to the throughput of the chromatographic procedure.

2.7. Calibration curves

Calibration curves were constructed by analyzing a series of plasma and tissue samples spiked with amlodipine racemate to obtain concentrations of the single amlodipine enantiomers ranging from 0.02 to 1.00 ng/ml and 0.2 to 9.6 ng/ml in plasma, from 2 ng to 60 ng/g in brain and from 50 to 600 ng/g in muscle. Samples were processed as described, and the chromatograms were evaluated on the basis of amlodipine/internal standard ratios of the peak heights. The calculation of the peak ratios was referenced to the diastereomer of the internal standard with the shortest retention time.

2.8. Extraction yields

The extraction yields of the (*R*)- and the (*S*)-enantiomer from plasma and tissue were evaluated by comparison of the results of normal runs of spiked plasma samples with runs of blank plasma samples where the same amount of amlodipine racemate was added to the first organic plasma extract.

2.9. Accuracy of enantiomer concentrations and enantiomeric ratios

A series of human plasma samples was spiked with different amounts of the (*R*)- and (*S*)-amlodipine, such that different concentration ratios of (*R*)/(*S*)-amlodipine were established. The sum of the concentrations of (*R*)- and (*S*)-enantiomers was adjusted to total amlodipine concentrations of *ca.* 4 ng/ml in one series of samples, and of *ca.* 8 ng/ml in a second series of samples. All samples were run through the procedure as described under *sample preparation* and the concentrations of the enantiomers were evaluated on the basis of calibration curves which were established by use of amlodipine

racemate. Given and found enantiomer concentrations and enantiomeric ratios were compared.

2.10. Assay precision

The within-day precision of the assay was determined by subsequential analysis of multiple spiked samples of amlodipine racemate in human plasma (two concentrations, $n = 6$), rat brain (one concentration, $n = 6$) and rat muscle (one concentration, $n = 8$). Additionally within-day precision of the assay in human plasma was characterized on the basis of duplicate estimations of samples from clinical investigations containing different amounts of the drug. Precision was expressed as mean coefficients of variation over the observed range of concentrations.

The day-to-day precision of the assay in human plasma samples was also estimated both from spiked standard samples as well as from double estimations of study samples analyzed on different days. The day-to-day variation of the assay in brain tissue was obtained from eight, and in muscle from seven spiked samples examined on seven consecutive days.

2.11. Biological samples

Amlodipine racemate (20 mg) was orally administered to a 34-year-old male healthy volunteer on an empty stomach. A pre-dose blood sample (8 ml) was obtained from the cubital vein immediately before ingestion of the drug. Further blood samples (8 ml each) were drawn in heparinized tubes at 1, 2, 4, 6, 8, 10, 12, 16, 24, 48, 72, 96, 120, 144, 168 and 192 h after administration. The blood was processed to plasma by centrifugation at 1500 *g*.

Amlodipine racemate (0.25 mg/kg) in 5% glucose solution was injected into the Vena saphena of a male beagle dog. Blood samples (5 ml) were collected in heparinized tubes immediately before and at 0.17, 0.34, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72 h after injection. Plasma was obtained as described above.

Racemic amlodipine besylate (1 mg/kg) in 5% glucose solution was intravenously injected into a single rat. After 30 min the rat was sacrificed and blood and tissue samples were taken.

3. Results and discussion

For the development of the present assay the following principal points had to be considered. Amlodipine is not volatile enough to be gas chromatographed without derivatization. Even derivatives such as N-trimethylacetylamlodipine [6] require elution temperatures which exceed the range of stability of the available chiral phases. Therefore it was necessary to employ an indirect method to separate the enantiomers, *i.e.* derivatization with a chiral reagent. The selected chiral reagent (+)-MTPAC has two important advantages: stable amide derivates are formed [7] which can be assayed with high sensitivity by electron-capture detection [7] due to the presence of the trifluoromethyl group. Both advantages apply to both derivatives of amlodipine. We confirmed that solutions of the derivatives in ethyl acetate could be stored at −4°C for several weeks without any signs of loss or degradation in the chromatograms. The same stability was observed for the derivatized extracts from plasma and tissue. The electron-capture response was also improved. By the derivatization with (+)-MTPAC, amlodipine could be detected with a 2–3 fold greater sensitivity than after derivatization with trimethylacetyl-chloride, which does not donate an electronegative group.

Typical chromatograms of amlodipine enantiomers after extraction from plasma and tissue and after derivatization with (+)-MTPAC are shown in Figs. 2 and 3. Retention times of the MTPA derivatives (diastereomers) were 21.6 min for the (*R*)-enantiomer and 21.8 min for the (*S*)-enantiomer and there were no interfering peaks from control tissue or plasma. The retention times of the diastereomeric derivatives of the internal standard were 27.0 min and 27.2 min, respectively. Fig. 2C represents an example of a relatively high difference in the concentrations of the enantiomers, while Fig. 3C demonstrates only a marginal difference between (*R*)- and (*S*)-amlodipine, which can however clearly be detected.

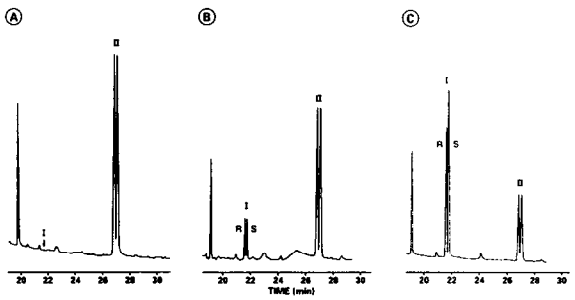

Fig. 2. Chromatograms of the products of chiral derivatization of amlodipine and internal standard enantiomers. (A) Blank human plasma; (B) human plasma spiked with 1 ng/ml amlodipine racemate; (C) plasma obtained at 24 h from a healthy volunteer after oral administration of 20 mg of amlodipine racemate. Peaks: I = amlodipine; II = internal standard.

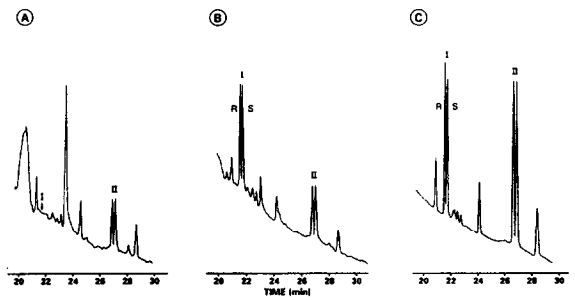

Fig. 3. Chromatograms of the products of chiral derivatization of amlodipine and internal standard enantiomers. (A) Blank rat tissue (brain); (B) rat tissue (brain) 0.5 h after i.v. administration of 1 mg/kg of amlodipine racemate; (C) rat tissue (muscle) 0.5 h after i.v. administration of 1 mg/kg of amlodipine racemate.

Good separation was achieved with a column of 100 μm I.D. and H_2 as carrier gas. The separation of chromatographic peaks can be judged on the basis of the resolution factor R [8]. R varies from zero (no resolution) to 1.5 (baseline or 100% resolution). The R of the diastereomeric pair of amlodipine derivatives was always above 1, corresponding to a peak separation better than 85%. Baseline separation of the diastereomeric pair was obtained on a polar 1301 stationary phase (6% Cyanopropyl phenyl, J&W Scientific). However, this type of column did not prove to be suitable for practical application, due to excessively long retention times (*ca.* 70 min) and corresponding peak broadening.

The enantiomers of amlodipine were extracted from plasma and tissue (Table 1) with a high yield (>80%) and reproducibility (coefficient of variation <9%). The values of (*R*)- and (*S*)-amlodipine were virtually identical.

The derivatization with (+)-MTPAC proceeded to completion under the conditions described under sample preparation. Extension of the reaction time up to 15 h and increase of the amount of reagent up to 10-fold did not result in increased formation of the diastereomers or in any change of their ratios.

For the application of the analysis to tissue, the sample preparation described for plasma was modified by the addition of clean-up steps. This was necessary in order to reduce matrix effects, which were manifested in lower extraction yields, particularly of the back-extraction into citric acid solution, and in chromatographic interferences. By UV-spectroscopic measurements it was confirmed that treatment of amlodipine solutions with the adsorbents XAD-2 and Carbopack C 0.8% THEED, under the

Table 1
Extraction recovery of amlodipine enantiomers in plasma and tissue

Biological matrix	Added amount of amlodipine racemate	*n*	Yield (mean ± S.D.) (%)	
			R	*S*
Plasma (man)	2.1 ng/ml	10	92.1 ± 7.5	93.0 ± 6.2
Brain (rat)	18.4 ng/g[a]	4	87.4 ± 6.8	87.0 ± 5.6
Muscle (rat)	345.0 ng/g[a]	6	83.0 ± 4.5	82.2 ± 4.8

[a] Wet weight.

Table 2
Calibration curves for amlodipine enantiomers

Biological matrix	*n*	Concentration range (ng/ml of plasma or ng/g of tissue[a])	Enantiomer	Slope	Intercept	Coefficient of correlation
Plasma (man)	1	0.02–0.10	*R*	5.5954	0.0381	0.9961
		0.02–0.10	*S*	5.4625	0.0613	0.9894
	3	0.1–9.6	*R*	0.2282	0.0134	0.9996
		0.1–9.6	*S*	0.2353	0.0118	0.9998
Brain (rat)	1	2–60	*R*	0.7969	0.0826	0.9986
		2–60	*S*	0.8301	0.0854	0.9986
Muscle (rat)	1	50–600	*R*	0.0328	0.0222	0.9998
		50–600	*S*	0.0335	0.0345	0.9997

Mean parameters of the linear regression (from *n* curves).
[a] Wet weight.

conditions described for the analysis, does not result in a loss of amlodipine.

Calibration curves of the amlodipine enantiomers were linear, and the regression parameters did not show systematic differences between the enantiomers (Table 2).

The lower limit of detection of the analysis was found to be 0.02 ng/ml in plasma and 0.5 ng/g in tissue, for each enantiomer. The assay sensitivity is sufficient to monitor drug levels and enantiomeric ratios of amlodipine even after the lowest therapeutic dose of 2.5 mg amlodipine racemate.

The precision of the method was also shown to be satisfactory. The within-day precision for spiked plasma samples was *ca.* 10% (coefficient of variation) of a concentration below 0.5 ng/ml and *ca.* 3% of a concentration above 2 ng/ml (Table 3), for both enantiomers. The mean coefficient of variation of duplicated estimations of clinical samples was also not higher than 10% over a range of measured enantiomer concen-

Table 3
Within-day precision of the assay of amlodipine enantiomers

Biological matrix	*n*	Enantiomer concentration (ng/ml of plasma or ng/g of tissue[a])		Coefficient of variation (%)	
		R	*S*	*R*	*S*
Plasma (man)	6	0.52	0.52	10.7	11.7
	6	5.23	5.23	2.6	2.8
	2	0.04–5.20 (35 samples)	0.13–5.24 (36 samples)	10.1[b]	6.2[b]
Brain (rat)	6	20.8	20.8	8.8	5.6
Muscle (rat)	8	352	352	2.3	4.1

[a] Wet weight.
[b] Mean coefficient of variation of double estimations.

Table 4
Day-to-day precision of the assay of amlodipine enantiomers

Biological matrix	n	Enantiomer concentration (ng/ml of plasma or ng/g of tissue[a])		Coefficient of variation (%)	
		R	S	R	S
Plasma (man)	10	1.14	0.52	10.7	11.7
	11	6.82	5.23	2.6	2.8
	2	0.63–6.75 (56 samples)	0.71–6.46 (56 samples)	11.1[b]	11.6[b]
Brain (rat)	8	20.8	20.8	9.0	10.2
Muscle (rat)	7	352	352	5.6	7.6

[a] Wet weight.
[b] Mean coefficient of variation of double estimations.

trations from 0.04 ng/ml to 5.24 ng/ml. The day-to-day precision of the assay in plasma was higher than the within-day precision for a comparable concentration range but was still of an acceptable order of magnitude (Table 4). Similar observations were made for the assay precision in tissue.

Table 5 gives data on the accuracy of the measured concentrations of (*R*)- and (*S*)-amlodipine in plasma samples at various concen-

Table 5
Accuracy of the assay of amlodipine enantiomers in plasma

Given			Found		
R (ng/ml)	S (ng/ml)	R/S ratio	R (ng/ml)	S (ng/ml)	R/S ratio
0.00	8.12	0.00	0.00	8.44	0.00
0.00	4.06	0.00	0.00	4.32	0.00
0.43	3.65	0.12	0.34	3.71	0.09
0.86	7.31	0.12	0.89	7.10	0.13
1.29	2.84	0.45	1.20	2.97	0.40
1.72	6.50	0.26	1.90	6.59	0.15
2.15	2.03	1.06	2.43	2.24	1.08
2.58	5.68	0.45	2.58	5.46	0.47
3.01	1.22	2.47	3.33	1.31	2.54
3.44	4.87	0.71	3.78	4.69	0.81
3.87	0.41	9.44	4.16	0.26	16.00
4.30	4.06	1.06	4.49	4.26	1.05
4.30	0.00	0.00	5.21	0.00	0.00
5.16	3.25	1.59	5.89	3.71	1.59
6.02	2.44	2.47	5.88	2.41	2.44
6.88	1.62	4.25	6.68	1.78	3.75
7.74	0.81	9.56	7.65	0.63	12.14
8.60	0.00	0.00	8.64	0.00	0.00

Samples were spiked with different amounts of (*R*)- and (*S*)-amlodipine resulting in various enantiomeric ratios of plasma concentrations.

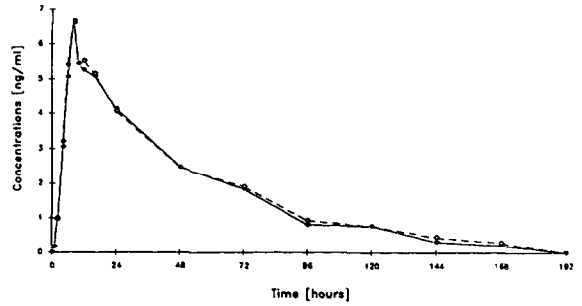

Fig. 4. Plasma concentrations of (*R*)-amlodipine (●) and (*S*)-amlodipine (○) in a healthy volunteer following oral administration of 20 mg amlodipine racemate.

tration ratios of the enantiomers. The recoveries were typically between 95% and 105% and were rarely outside the 90–110% range. There was no apparent difference in the assay accuracy between the two enantiomers.

The described enantioselective method was applied to the quantitation of amlodipine in biological samples. Fig. 4 shows the concentration–time curves of the amlodipine enantiomers in a healthy subject after an oral dose of amlodipine racemate. The measured concentrations of (*R*)- and (*S*)-amlodipine were practically equal over the whole period of observation. The plasma concentrations of the amlodipine enantiomers, measured in a dog following an intravenous dose of 0.25 mg/kg amlodipine racemate (Fig. 5), did not show pronounced enantioselectivity in the disposition of amlodipine

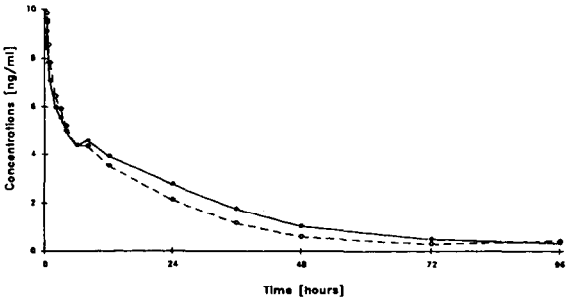

Fig. 5. Plasma concentrations of (*R*)-amlodipine (●) and (*S*)-amlodipine (○) in a beagle dog following i.v. injection of 0.25 mg/kg amlodipine racemate.

Table 6
Concentration of amlodipine enantiomers in the rat 30 min after 1 mg/kg racemate given intravenously to one animal

Body compartment	(*R*)-Amlodipine	(*S*)-Amlodipine
Lung	3705 ng/g[a]	3688 ng/g[a]
Heart	801 ng/g[a]	779 ng/g[a]
Muscle	386 ng/g[a]	351 ng/g[a]
Brain	24 ng/g[a]	23 ng/g[a]
Plasma	19 ng/ml	20 ng/ml

[a] Wet weight.

either. In rat, a high affinity of amlodipine for some tissues was observed, while the distribution was very similar for the two enantiomers (Table 6).

4. Conclusion

Our results demonstrate that the (+)-(*R*)- and the (−)-(*S*)-enantiomer of amlodipine can be converted into diastereomers and subsequently separated on an achiral capillary column when (+)-MTPAC is used as a chiral reagent. This method is applicable to extracts of biological material. The enantioselective assay is sufficiently sensitive and reliable to measure (*R*)- and (*S*)-amlodipine at concentrations as low as 0.02 ng/ml in plasma, and 0.5 ng/g in various tissues.

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