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Determination of α-methyldopa in human plasma by validated high-performance liquid chromatography with fluorescence detection

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Abstract

A sensitive reversed-phase gradient elution high-performance liquid chromatographic method with fluorescence detection has been developed for the determination of α-methyldopa (AMD) in human plasma. Separation of the investigated compound and the 3,4-dihydroxyphenylalanine (DOPA) internal standard was achieved on a Nucleosil 7 C_{18} column with a 5 m*M* heptanesulphonic acid sodium salt containing 0.05 *M* potassium dihydrogenphosphate (pH 3.2)–acetonitrile mobile phase. The composition of the mobile phase was changed according to a linear gradient time program. Detection was performed at 270 nm fluorimetric excitation and 320 nm emission. The compounds were isolated from plasma by Bond-Elut C_{18} solid-phase extraction. The limit of quantitation was found to be 10 ng/ml plasma. The assay was validated with respect to accuracy, precision and system suitability. All validated parameters were found to be within the 20% required limits. On the basis of the sensitivity, linearity and validation parameters the developed analytical method was found to be suitable for application in a bioequivalency study.

Keywords: Methyldopa; DOPA

1. Introduction

The aromatic L-aminodecarboxylase inhibitor α-methyldopa (AMD) has become one of the most widely used antihypertensives since the original publication of Oates et al. in 1960 [1].

AMD undergoes mainly decarboxylation and 3-O-methylation. The main metabolites are 3-O-methyl-α-methyldopa, α-methyldopamine, 1-O-methyl-α-methyldopamine, α-methylnoradrenaline and 3,4-dihydroxyphenylacetone [2]. α-Methyldopamine and α-methylnoradrenaline are biologically active. Sulphate conjugation of AMD is also a possible metabolic pathway [3].

Spectrofluorimetric [4–6], gas and liquid chromatographic [7, 10–12] methods are used for the quantitative determination of AMD in biological samples. High-performance liquid chromatographic systems equipped with electrochemical detectors are preferentially used nowadays [8,9].

The aim of our study was the development of an HPLC method with fluorescence detection for the determination of AMD in human plasma samples and validation of the analytical procedure.

For the analytical method to fit the purpose of bioequivalency studies of AMD containing drugs, the detection limit must be 10 ng AMD/ml plasma sample.

We used solid-phase extraction for sample preparation followed by reversed-phase ion-pair chroma-

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tography. The internal standard was 3,4-dihydroxyphenylalanine (DOPA).

2. Experimental

2.1. Materials

α-Methyl-3,4-dihydroxyphenylalanine (AMD) and the internal standard L-3,4-dihydroxyphenylalanine (DOPA) (Sigma, St. Louis, MO, USA) were provided by EGIS Pharmaceuticals (Budapest, Hungary). The structural formulae of the substances are shown in Fig. 1. Methanol and acetonitrile (HPLC grade) were produced by Chemolab (Budapest, Hungary). The Bond-Elut C_{18}, 1-ml solid-phase extraction cartridges were manufactured by Varian (Harbor City, CA, USA). Heptanesulphonic acid sodium salt was purchased from Sigma. Potassium dihydrogenphosphate, EDTA-Na and 85% phosphoric acid (all analytical grade) were from Reanal (Budapest, Hungary), hydrochloric acid (Ph.Hg.VI.) was purchased from Finomvegyszer Szövetkezet (Budapest, Hungary). Perchloric acid 70% (analytical grade) was produced by Laborchemie (Germany).

2.2. Chromatographic conditions

LC-6A pumps, a RF-551 fluorimetric detector and a C-R6A integrator were used. Injection was performed with a SIL-6B automatic sample injector (equipped with SCL-6B system controller) through a 100-μl loop. All instruments were Shimadzu (Kyoto, Japan) products.

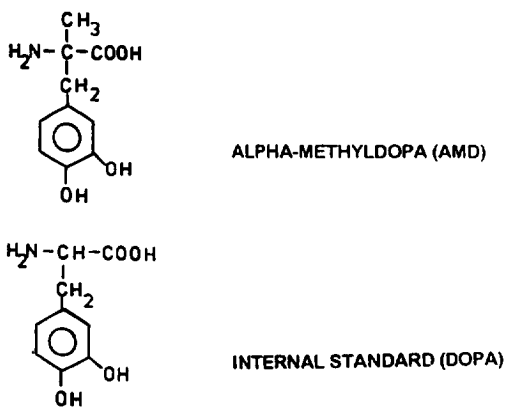

Fig. 1. Chemical structures of the investigated compounds.

Separation was performed at room temperature (air-conditioned room 20±2°C) on a Nucleosil 7 C_{18} (250×4 mm I.D.) column equipped with a Nucleosil 7 C_{18} (30×4 mm I.D.) guard column (BST Budapest, Hungary). The flow-rate was 1.0 ml/min.

The fluorimetric excitation wavelength was 270 nm, and the emission wavelength was 320 nm. The mobile phase consisted of: solvent A: 5 m*M* heptanesulphonic acid sodium salt containing 0.05 *M* potassium dihydrogenphosphate (pH 3.2, adjusted with 1 *M* phosphoric acid)–acetonitrile (95:5, v/v). The eluent was filtered through a 0.45-μm Nylon 66 membrane (Supelco, Bellefonte, PA, USA) and degassed with nitrogen; solvent B: acetonitrile.

The composition of the mobile phase was changed according to the following time program: 0–15 min, 100% A, 0% B, isocratic; 15–18 min: linear gradient from 100% to 25% A, and 0% to 75% B; 18–25 min: 25% A, 75% B, isocratic; 25–32 min: linear gradient from 25% to 100% A, and 75% to 0% B; 32–45 min: 100% A, 0% B, isocratic.

2.3. Solutions

The 1 mg/ml stock solution of AMD was prepared with 0.2 *M* perchloric acid. Dilutions for the working solutions were also made with 0.2 *M* perchloric acid (1, 10, and 100 μg/ml).

The internal standard (DOPA) stock solution (1 mg/ml) and the working solutions were also made with perchloric acid (10 μg/ml).

The stock solutions were stable for 1 month stored in the refrigerator at −20°C. Working solutions were freshly prepared each week and stored in a refrigerator at 4°C.

Human plasma was obtained from blood samples collected from the cubital vein. Coagulation was prevented by adding 15 mg of EDTA-sodium salt to 15 ml of blood. The anticoagulant was dissolved in 0.15 ml of distilled water. Blood treated with anticoagulant was centrifuged at 1500 *g* for 10 min and

the obtained drug-free plasma was stored at −20°C until use.

2.4. Plasma extraction

To 1 ml of plasma 1000 ng internal standard (from 10 μg/ml 0.2 *M* perchloric acid solution in 100 μl volume) and 1 ml of 1 *M* hydrochloric acid were added. The sample was homogenized by 5 s vortex-mixing, then poured onto Bond-Elut C_{18} (1 ml) solid-phase extraction column previously activated with 1 ml of methanol and 1 ml of 0.25 *M* hydrochloric acid. The column was washed with 0.2 ml bidest. water and dried with a stream of air. After 5 min elution was performed with 0.3 ml of 0.2 *M* perchloric acid–acetonitrile (9:1, v/v). Aliquots (100 μl) of the eluate – without evaporation – were injected onto the analytical column.

For calibration, 'spiked' model samples were made from pooled drug-free plasma by adding the actual amount of diluted stock solution (1, 10, or 100 μg/ml 0.2 *M* perchloric acid solution) together with the internal standard.

2.5. Method validation

2.5.1. Precision of the chromatographic system

Both compounds were injected five times from their own working (10 μg/ml) solutions. The concentration of the working solutions is the following: 100-μl loop=500 ng of DOPA and 500 ng of AMD)/injection.

2.5.2. Summary of the data of the calibration curves

To 1 ml of drug-free plasma increasing amounts of AMD (0, 10, 100, 250, 1000, 3000 ng) and 1000 ng of internal standard (DOPA) were added.

Extraction and liquid chromatography were carried out as previously described. The ratio of the peak areas of the investigated compound (AMD) and the internal standard (DOPA) were plotted against plasma concentration. The calibration curves were fitted to the measured points by the weighted least squares method (weighting factor $w=1/y^2$) using an HP 85 B (Hewlett-Packard) computer.

2.5.3. Intra-day precision

Amounts of 35, 200, and 2000 ng of AMD and 1000 ng of internal standard were added to 1 ml of plasma. Analysis was performed with 6 samples at each concentration.

2.5.4. Between-day precision

Amounts of 10, 100, 250, 500, 1000, and 3000 ng of AMD and 1000 ng of internal standard were added to 1 ml of plasma. The determinations were made on 2×3 consecutive days.

2.5.5. Determination of extraction efficiency

Amounts of 10, 100, 250, 500, 1000, and 3000 ng of AMD were added to 1 ml of plasma. Solid-phase extraction was performed without internal standard, 1000 ng of internal standard was added to the perchloric acid–acetonitrile eluate after the extraction. The peak area ratios were compared to the ratio of the standard aqueous samples without extraction. Five replicate samples were determined at each point.

2.5.6. Stability

The stability of the 25 and 1000 ng of AMD/ml plasma samples stored at −20°C was studied after 16 and 28 days of storage. The internal standard was added immediately before the extraction of the samples.

Three parallel determinations were made in each case and at each time.

3. Results and discussion

We have developed an HPLC method for the quantitative determination of AMD from human plasma by fluorescence detection The excitation wavelength of 270 nm and the emission wavelength of 320 nm are the fluorescent maximum values for AMD.

By using a Nucleosil 7 C_{18} column and the mobile phase of 5 m*M* heptanesulphonic acid sodium salt/0.05 *M* potassium dihydrogenphosphate–acetonitrile (95:5, v/v) almost ideal peaks were obtained which were symmetrical for both the compound to be determined and the internal standard (Fig. 2). The

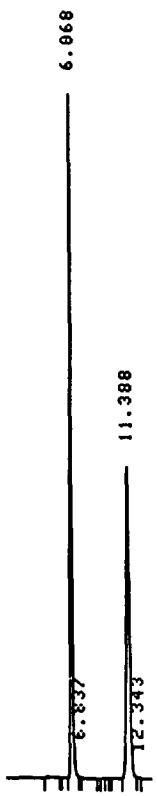

Fig. 2. Chromatograms of 500 ng DOPA and 500 ng AMD. DOPA t_R=6.068 min, AMD t_R=11.388 min (for chromatographic conditions see text).

reproducibility of the chromatographic conditions (retention time, peak area) was determined by replicate injections (n=5) of 100-μl standard samples containing 500 ng of DOPA and 500 ng of AMD. Based on the system suitability test, the relative standard deviations for peak areas of DOPA and AMD were found to be 1.22% and 1.03%, respectively. The mean retention times for DOPA and the AMD were 6.09(±0.054) and 11.54(±0.11) min, respectively.

Among the Bakerbond C_{18} (Baker, Phillipsburg, NJ, USA), Sep-Pak C_{18} (Waters, Milford, MA, USA), Samplex C_{18} (BST, Budapest, Hungary) and Bond-Elut C_{18} (Varian) solid-phase extraction columns the latter was the best for the measurements.

Fig. 3 shows chromatograms of solid-phase extraction by Bond-Elut C_{18} (1 ml) resulting in typical blank plasma extracts (Fig. 3A and C) as well as the spiked sample chromatograms of 250 ng of AMD and 1000 ng of internal standard in 1 ml of plasma (Fig. 3B and D). It can be clearly seen from the chromatograms of the typical blank and the 250 ng AMD and 1000 ng DOPA containing plasma samples (Fig. 3A and B) obtained after 90 min isocratic (100% A mobile phase) procedure that there are no interfering endogenous substances in the retention range of AMD and DOPA. Under isocratic conditions, however, a wide endogenous peak was observed at approx. 70 min. This and the other peak at 8.5 min did not disturb the actual detection but prolonged the duration of the analysis (90 min).

Since we have aimed at the development of a method suitable for clinical pharmacological serial determinations, efforts were to shorten the analysis time. In order to achieve this, an acetonitrile gradient was applied after the 15-min isocratic analysis (see Section 2.2). We consider this isocratic HPLC method, where, after the analysis procedure (15 min), the solvent strength was increased in order to rapidly elute the endogenous substances from the analytical column. Thus the total analysis time is 35 min.

The gradient eluted chromatograms of a typical blank (Fig. 3C), and those of samples containing 250 ng of AMD and a 1000 ng of DOPA internal standard (Fig. 3D) are also shown. There are no interfering endogenous peaks in the case of gradient elution, and reinjection is possible after 35 min.

The detailed validation data are shown in Tables 1–3. The reproducibility of the method is within the accepted limit of 20% R.S.D. According to the validation the limit of quantitation of AMD was 10 ng/ml plasma and the limit of detection was 5 ng/ml plasma, respectively. The calibration curves of the area ratios versus the concentration of the investigated compound determined by the regression model with $w=1/y^2$ were linear in the 10–3000 ng/ml plasma concentration range [$y=0.01155+2.2519\cdot 10^{-3}x$, $R^2=0.999$; where y is the average ratio of the peak areas of the investigated compound and the internal standard, and x is the concentration of the investigated compound (ng/ml plasma)]. The data of the calibration curves are summarized in Table 1.

The R.S.D. of the slopes of the calibration curves were 2.79%.

Table 2 demonstrates the intra-assay (intra-day) and inter-assay (between-day) precision and accura-

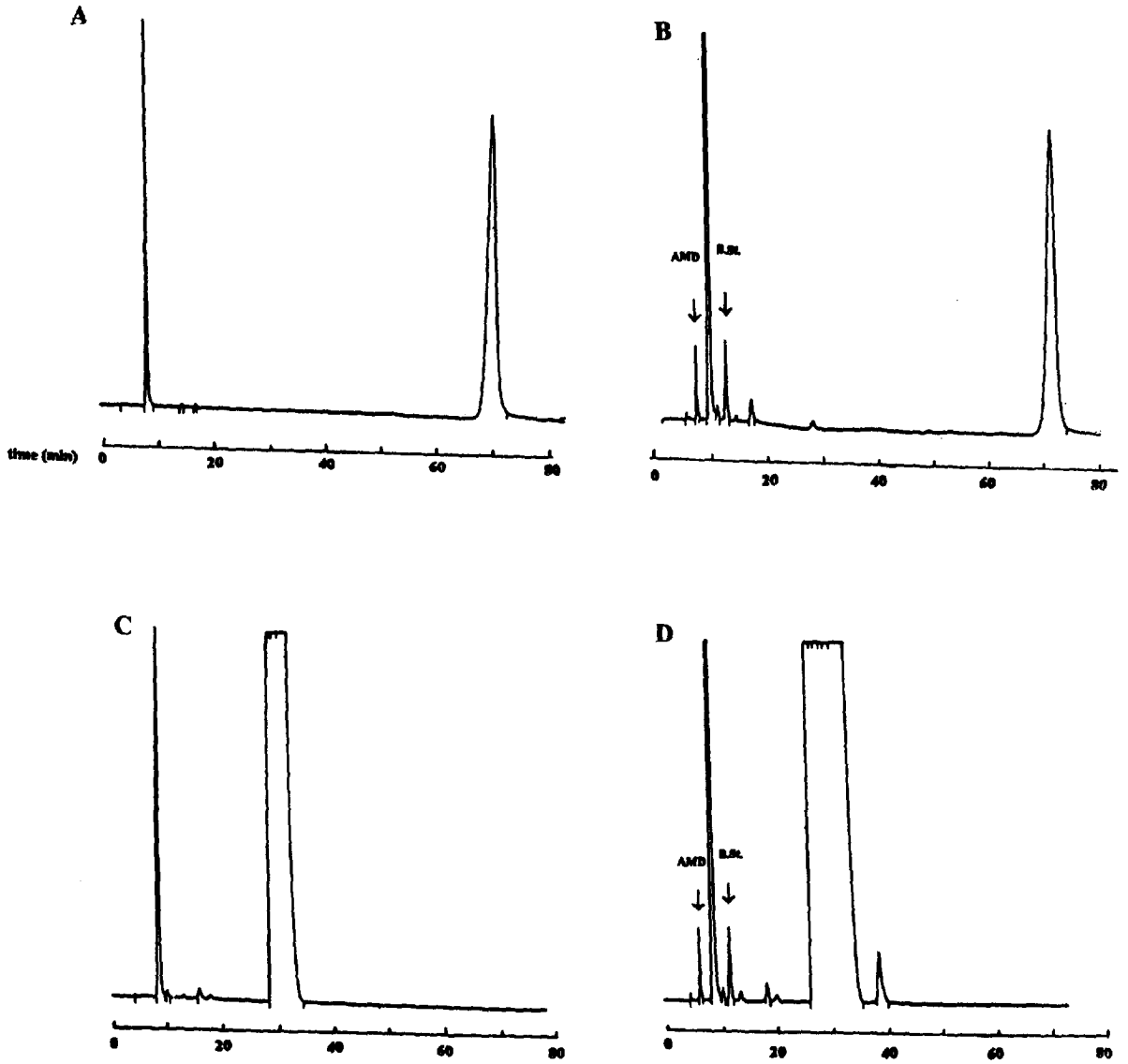

Fig. 3. Isocratic chromatogram of a typical blank plasma sample (A) and of a 250 ng AMD and 1000 ng DOPA internal standard/ml plasma model sample (B). Gradient chromatogram of a typical blank plasma sample (C) and a 250 ng AMD and 1000 ng DOPA/ml plasma sample (D) (for chromatographic conditions see text).

cy of the method. The average R.S.D. of the intra-assay analysis was 5.66%. Acceptable accuracy was achieved for all concentrations investigated. The inter-assay precision was calculated from data obtained on six different days. The average R.S.D. was 7.96%. The accuracy of the assays ranged from 95.6% to 109.02%, which are acceptable values for biological samples.

The results of the comparison of neat standards versus plasma extracted standards of AMD in the concentration range 10–3000 ng/ml indicated that the extraction recovery of AMD from human plasma was independent of the AMD concentration. The average recovery was 94.02% for AMD ($n=5$ at each concentration).

According to the experiments, AMD as well as the

Table 1
Summary of the calibration curves

Added concentrations (ng/ml)	AMD/DOPA peak-area ratios (mean ± S.D.)	R.S.D. (%)	n
10	0.034±0.0064	18.82	6
100	0.240±0.038	15.83	6
250	0.581±0.091	15.66	6
500	1.132±0.154	13.60	6
1000	2.241±0.273	12.18	6
3000	6.720±0.279	4.15	6

Table 2
Precision

Nominal concentration	Measured concentrations (mean±S.D.) (ng/ml)	Accuracy (%)	R.S.D. (%)	n
Intra-day				
35	35.3±2.5	100.86	7.12	6
200	216.3±11.1	108.15	5.13	6
2000	2072±98.5	103.62	4.75	6
Average			5.66	
Between-day				
10	10.9±2.0	109.02	18.35	6
100	95.6±10.3	95.60	10.74	6
250	247.3±17.8	98.92	7.20	5
500	505.8±30.2	101.16	5.97	6
1000	1013.2±48.0	101.32	4.73	6
3000	2999.5±24.7	99.98	0.82	6
Average			7.96	

internal standard in stock solution remained stable for 1 month.

AMD in human plasma showed no significant degradation during 4 weeks storage at −20°C. The accuracy of AMD in plasma after storage was between 95.64 and 107.48% (Table 3).

This analytical procedure provides a relatively simple and sensitive method for the determination of AMD in human plasma. The usefulness of the method has been demonstrated by its application to the analysis of plasma samples in bioequivalency studies.

Table 3
AMD stability in human plasma during storage at −20°C

Number of storage days	Added AMD (ng/ml)	Measured AMD (mean±S.D.) (ng/ml)	Accuracy (%)	R.S.D. (%)	n
16	25	26.87±3.1	107.48	11.53	3
	1000	1040.2±50.9	104.00	4.89	3
28	25	23.91±2.8	95.64	11.71	3
	1000	1062.2±69.2	106.22	6.51	3

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