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DETERMINATION OF METHYLDOPA IN HUMAN PLASMA BY LC/MS-MS FOR THERAPEUTIC DRUG MONITORING

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ABSTRACT. A simple and rapid liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) method for therapeutic level monitoring of methyldopa in human plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a mobile phase of 2:98 (v/v) acetonitrile and 0.2% (v/v) formic acid in water at 40ºC with a flow rate of 0.8 mL/min. The detection of was performed using an ion trap MS with electrospray positive ionisation in multiple reaction monitoring (MRM) mode (m/z 212.1 →139.2, 166.2, 195.2). The human plasma samples (0.2 mL) were deproteinised with methanol and aliquots of 1.5 µL from supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity, precision (CV < 8.4 %) and accuracy (bias < 11.1 %) over the range of 0.32-20.48 µg/mL plasma. The lower limit of quantification (LLOQ) was 0.32 µg/mL and the recovery was between 90.9-101.4 %. The method is fast, with a minimum time for plasma sample preparation and a run-time of 1.5 min for instrument analysis (retention time of methyldopa was 1.05 min). The developed and validated method is simple, rapid, selective and sufficiently sensitive to be applied in clinical level monitoring, pharmacokinetics or bioequivalence studies of methyldopa.

Keywords: *methyldopa, LC/MS-MS, therapeutic drug monitoring*

INTRODUCTION

Methyldopa, 2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid, (Fig. 1), is an antihypertensive drug with central action [1]. It stimulates the central alpha-2-adrenoreceptors, primarily by its metabolite alpha-methyl-norepinephrine, and decreases sympathetic outflow and blood pressure [2, 3].

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Methyldopate hydrochloride is another drug also used in antihypertensive therapy. It is the ethyl-ester of methyldopa, so a prodrug thereof, becoming pharmacologically active through its metabolism to methyldopa [1].

The chemical structure of methyldopa is analogue of dopa (dihydroxy-phenylalanine), from which it differs through the presence of a methyl group on the alpha-carbon of the side chain, containing thus a chiral centre (Fig. 1). The S-isomer is responsible for antihypertensive activity [2, 4].

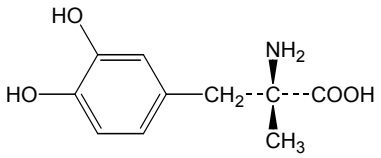

Figure 1. Chemical structure of methyldopa

After oral administration, the absorption of methyldopa is approximately 42% (± 16%). The peak plasma levels occur in 2–4 h and the therapeutic plasma concentrations are usually in the range 1-5 μg/mL. The plasma protein binding is < 20%. The drug is excreted in the urine as decarboxylated metabolites, sulphate conjugate, and unchanged drug [1, 3]. About 40% of the oral dose is excreted in the urine in 48 h, of which about 40% is the conjugate, and a considerable amount of unchanged drug is eliminated in the faeces. Methyldopa has a half-life of about 2 h, but a longer terminal elimination half-time has also been reported [1]. After IV administration of methyldopate, the bioavailability of methyldopa is similar to oral administration because a large portion of ester is not hydrolyzed to methyldopa. Between 52-82% of a IV dose is excreted in the urine in 36 h, and only ~2% is conjugated [1, 3].

Methyldopa is not generally used as a first-line drug in antihypertensive therapy due to its frequent side effects, as sedation and drowsiness, and also sympathetic depression. But it lowers blood pressure without compromising renal blood flow or the glomerular filtration rate, being preferred especially in hypertensive patients with renal complications [2]. It also represents one of the drugs frequently used in pregnancy to treat non-severe hypertension [5, 6]. Therapeutic drug monitoring is required in these cases to ensure the effectiveness of drug treatment and to avoid possible adverse effects.

Several methods involving spectrofluorimetry [7] and high-performance liquid-chromatography (HPLC) with UV [8, 9], fluorescence [7, 9-11], electrochemical [12] or mass spectrometry (MS) [13] detection has been reported to determine therapeutic levels of methyldopa in biological samples: serum [9, 10] or plasma [7, 8, 11-14], urine [7, 12], breast milk [14]. Several of these methods are long and include extraction procedures that prolong the time and cost of analysis.

Generally, the sensitivity of native fluorescence detection of catecholamines, including methyldopa, is three orders of magnitude lower than those detectable by UV. Greater sensitivity is obtained by fluorescence detection after derivatisation, but the time of analysis is longer due to derivatisation steps required [9]. Electrochemical detection offers good sensitivity, however it includes long separation times and usually needs quite complex extractions [12].

In the last decade, mass spectrometry has become the detection mode preferred for liquid chromatography due to its powerful performances: it allows the identification of separated compounds with high accuracy and sensitivity, particularly selective determination, and robustness [15-20]. Sample preparation is commonly simple and rapid and often requires only precipitation of proteins (PP) before chromatographic analysis [13, 17, 20].

The aim of this work was to develop and validate a high-throughput LC/MS-MS method to quantify methyldopa levels in human plasma. This method will be applied in therapeutic drug monitoring and bioavailability studies.

RESULTS AND DISCUSSION

The developed LC/MS-MS method was optimized and validated. It is rapid, with a total run time of instrumental analysis of 1.5 min and a retention time of methyldopa of 1.05 min (Fig. 2). Sample preparation was short including only the precipitation of proteins and processing a small volume of plasma (0.2 mL). The sensitivity was good (LLOQ of 0.32 µg/mL), sufficient to determine therapeutic levels of methyldopa ranging between 1-5 µg/mL. The absolute recoveries were high (between 90.9±6.3 al LLOQ and 99.3±6.1 at 10.24 µg/mL, respectively).

Sample preparation

In the developed method volumes of only 0.2 mL plasma were precipitated with methanol (0.6 mL) and analysed by direct injection of centrifuged supernatant into the chromatographic system. This method is more rapid and offers a shorter time of analysis, and thus a lower cost of routine determinations as compared to the other methods reported in literature (Table 1).

The method involved in sample preparation can influence the chromatographic background level and generate matrix suppression effect in LC-MS assays. No matrix interference or ion suppression was observed from the plasma samples in the developed method. In the scientific literature, there were reported some methods that also used protein precipitation (with perchloric acid or methanol) without extraction for the determination of methyldopa

in human plasma [8, 9, 13]. Several researchers prefer to include in plasma sample preparation an extraction step to eliminate the impurities and to increase the sensitivity (Table 1). Due to the amphoteric nature of methyldopa that would make the isolation difficult by simple liquid-liquid extraction (LLE), solid-phase extraction (SPE) or/and alumina extraction are preferred as isolation methods [10-12]. But the extraction steps increase the time of analysis and the costs of routine determinations, and can affect the recovery.

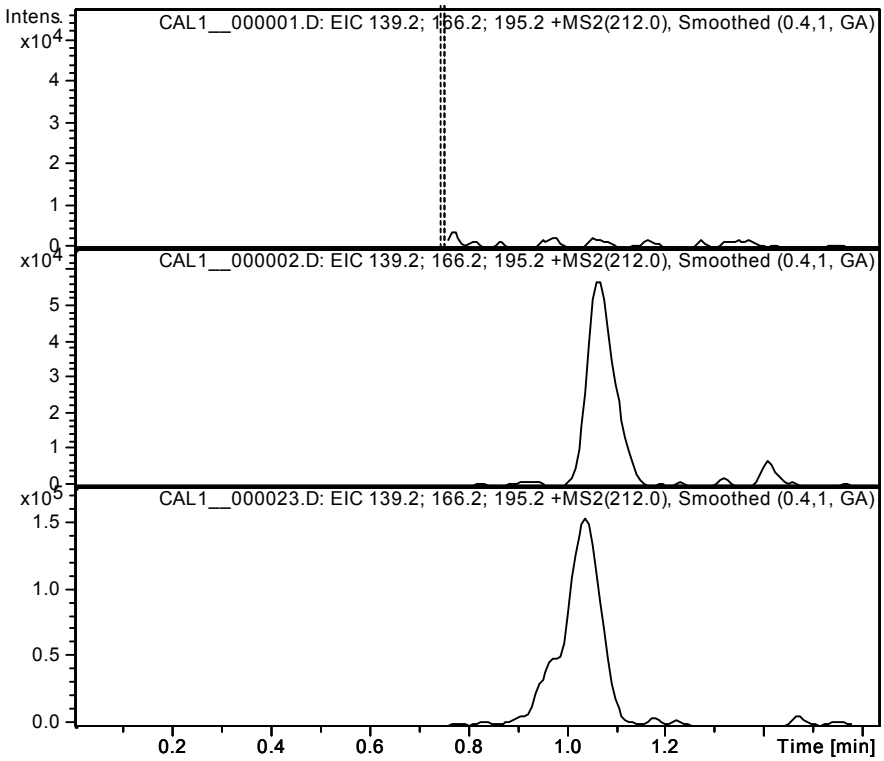

Figure 2. Representative chromatograms of (up) drug-free plasma, (middle) plasma spiked with methyldopa at LLOQ (0.32 μg/mL) and (down) plasma sample obtained from a patient 60 min after administration of methyldopa (concentration found: 1.12 μg/mL).

Li *et al.* used HPLC with diode array detection (DAD) combined with second-order calibration based on alternating trilinear decomposition algorithm for simultaneous quantitative analysis of methyldopa, levodopa and carbidopa. They obtained a good sensitivity (LOQ = 0.244 μg/mL), and a good linearity for the studied range of methyldopa (0.0-7.0 μg/mL), but a relatively long retention time of 3.46 min [8].

Only a LC-MS/MS assay is reported for quantification of methyldopa in human plasma after protein precipitation with perchloric acid. The quantification was performed in MRM mode (m/z 212.1→166.1). Methyldopa has a retention time of 3.4 min and the authors obtained a LOQ of 20 ng/mL. But the recovery was poorer in comparison with our method (93±5, 89±7 and 83±11% for 0.025, 0.25 and 2.5 μg/mL, respectively) [13].

Table 1. Analytical characteristics of some reported HPLC methods for the determination of methyldopa in human plasma or serum

Ref.	Matrix (mL)	Pre-treatment/ extraction[c]	Stationary phase	Mobile phase constituents[b]	Detection mode[a]	LOQ[d] (μg/mL)	Rt[e] (min)	Absolute recovery (%)
Our method	Plasma (0.2)	PP with methanol	Zorbax SB-C18	ACN: 0.2% (v/v) formic acid (2:98,v/v)	ESI-MS/MS, MRM, m/z 212.1→(139.2, 166.2, 195.2)	0.32	1.05	90.9-101.4
Oliveira [13]	Plasma (0.2)	PP with $HClO_4$	Genesis C18	10 mM ammonium acetate buffer (pH 5)-MeOH (70:30,v/v)	ESI-MS/MS, MRM (m/z 212.1→166.1)	0.02	3.4	88
Li [8]	Plasma (0.25)	PP with methanol	Hypersil-ODS	MeOH-0.002M KH_2PO_4 (pH 5) (25:75, v/v)	DAD + SOC-ATLD	0.244	3.46	104±2.6
Muzzi [9]	Serum (0.25)	PP with $HClO_4$	Supercosil LC-18	MeOH-0.02M KH_2PO_4 (pH 2.5), gradient	FD	105 pmol/mL	14.5	NA[f]
				50 mM Tris (pH 7)-MeOH - ACN (8:1:1)	FD with derivatisation	50 pmol/mL		
Bahrami [10]	Serum (1.0)	Alumina extraction	Shimpack-CLC-ODS	MeOH-0.05M KH_2PO_4 (+ TEA, pH 2.3) (8:92, v/v)	FD	0.02	1.7	98±3
Rona [11]	Plasma (1.0)	SPE	Nucleosil 7 C18	5 mM HSA-Na salt containing 0.05 M KH_2PO_4 (pH 3.2)-CAN, gradient	FD	0.01	6.068	94

[a] DAD, diode array detection; SOC-ATLD, second-order calibration based on alternating trilinear decomposition algorithm; FD, fluorescence detection; MRM, multiple reaction monitoring; [b]MeOH, methanol; ACN, acetonitrile; TEA, triethylamine; HAS, heptansulfonic acid; [c]PP, protein precipitation; SPE, solid phase extraction; [d]LOQ, limit of quantification; [e]Rt, retention time; [f]NA, not available.

LC-MS assay

The chromatographic conditions, especially the composition of the mobile phase, were optimized in several trials to achieve maximum peak responses and symmetrical chromatographic peaks, a short retention time of methyldopa

and consequently a shorter run time of analysis. The best results were obtained with the mixture of acetonitrile and 0.2% (v/v) formic acid in water (2:98, v/v) under isocratic conditions.

The electrospray ionization (ESI) in positive ion mode offers significantly higher signals for methyldopa compared to ESI in negative ion mode or atmospheric pressure chemical ionization (APCI). The direct MS detection is used for pharmaceutical purposes in qualitative rather than quantitative analysis. The use of tandem MS detection allows the obtention of better selectivity and sensitivity by the fragmentation of the molecular ion into several ions. The molecular ion $[M+H]^+$ (m/z 212.2) of methyldopa was fragmented into three abundant ions (m/z 195.2, 166.2, 139.2) at the optimum collision energy of 1.2 V (Fig. 3). The detection was carried out in multiple reaction monitoring (MRM) and the extracted ion chromatogram (EIC) of m/z (195.2, 166.2, 139.2) from m/z 212.2 was monitored and analyzed.

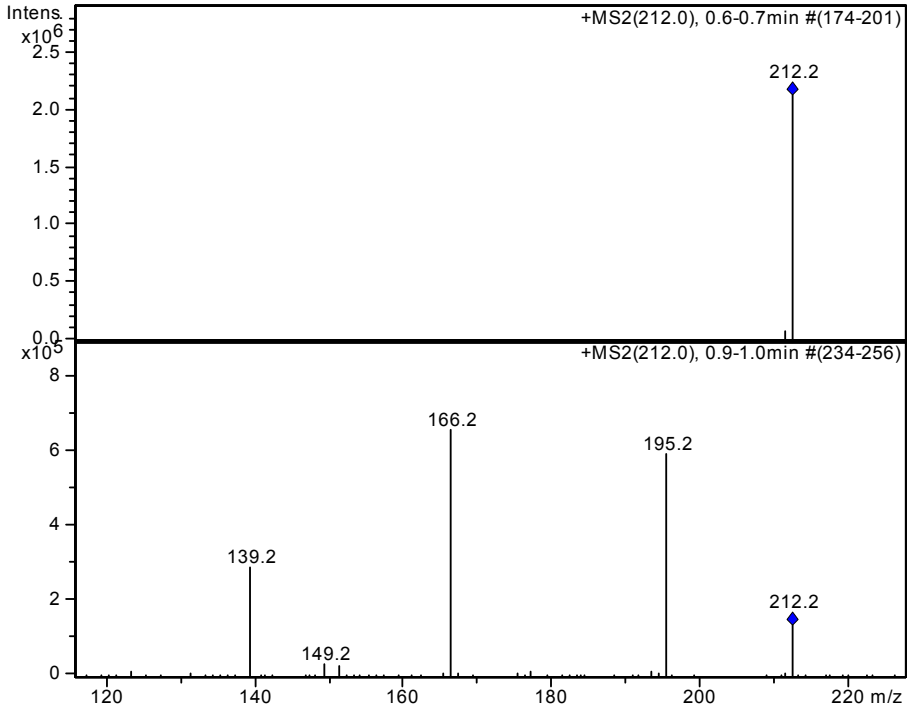

Figure 3. Mass spectra of methyldopa obtained by electrospray ionisation in positive ion mode at the collision energy of 1.2 V: (up) full-scan spectrum with molecular ion $[M+H]^+$ (m/z 212.2); (down) MS/MS reactive spectrum (after fragmentation into monitored ions: m/z 195.2, 166.2, 139.2).

Table 2. The intra-day precision (CV %), accuracy (bias %) and recovery data for the measurement of methyldopa in human plasma (the analysis of five different samples, n = 5)

Nominal concentration	Found concentration mean		CV	Bias	Recovery	
(μg/mL)	μg/mL	± SD	(%)	(%)	(%)	± SD
0.32	0.33	0.02	5.3	2.2	93.7	5.0
0.64	0.61	0.02	2.6	-4.5	95.6	11.1
2.56	2.59	0.12	4.5	1.2	97.0	3.1
10.24	11.38	10.37	3.3	11.1	101.4	2.8

Table 3. The inter-day precision (CV %), accuracy (bias %) and recovery data for the measurement of methyldopa in human plasma (one analysis on five different days, n = 5)

Nominal concentration	Found concentration mean		CV	Bias	Recovery	
(μg/mL)	μg/mL	± SD	(%)	(%)	(%)	± SD
0.32	0.33	0.02	7.6	2.7	90.9	6.3
0.64	0.62	0.02	2.8	-2.3	94.2	5.1
2.56	2.48	0.21	8.4	-3.2	101.0	4.1
10.24	10.95	0.47	4.3	6.9	99.3	6.1

Assay validation

The method was validated in accordance with international regulations [21, 22]. Representative chromatograms of drug-free plasma and plasma spiked with methyldopa at LLOQ are shown in Fig. 2. No interfering peaks from the endogenous plasma components were observed in the retention time of methyldopa.

The calibration curves were described by a quadratic equation over the concentration range of 0.32 – 20.48 μg/mL in human plasma, with a correlation coefficient greater than 0.99. The LLOQ was 0.32 μg/mL, being the lowest concentration having an intra- and inter-day CV and bias under 20% [21]. The values obtained for intra-day and inter-day precision and accuracy during the validation are shown in Tables 2 and 3, respectively.

All values for accuracy and precision were within recommended limits (<15%). The means of absolute recovery values were between 90% and 101%.

Method application

The validated method was used for therapeutic drug monitoring of methyldopa in pregnant women treated for hypertension. Blood sampling was made at 60 min after drug administration (starting with 1g methyldopa daily) and the mean found concentration was 1.81±0.89 μg/ml (n=14). The drug

plasma levels under 0.9 μg/ml were clinically correlated with an insufficient decrease of arterial tension and the daily methyldopa dose was increased to 1.5 or 2 g.

CONCLUSION

The developed and validated LC-MS/MS method satisfied the requirements of a high-throughput assay by its sensitivity, specificity, speed and simplicity. In comparison with other published HPLC [8-12, 14] or LC-MS/MS [13] methods for therapeutic level monitoring of methyldopa in human plasma, the developed method performs better in terms of volume of analyzed plasma sample, analyte recovery, and rapidity (both sample preparation and chromatographic run-time), which are essential attributes for methods used in routine analysis. The method can be successfully applied in bioequivalence or pharmacokinetics studies, or for therapeutic level monitoring of methyldopa.

EXPERIMENTAL SECTION

Reagents

Acetonitrile and methanol of isocratic grade for liquid chromatography, and formic acid of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Deionised water was obtained using a Milli-Q Water purification system (Millipore, Milford, MA, USA). The human blank plasma was supplied by the Regional Blood Transfusion Centre of Cluj-Napoca (Romania) from healthy volunteers, men and women.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus and Precision Standard Balance (Mettler-Toledo, Switzerland); Vortex Genie 2 mixer (Scientific Industries, New York, USA); Ultrasonic bath Elma Transsonic 700/H (Singen, Germany). The HPLC system used was an 1100 series Agilent Technologies model (Darmstadt, Germany) consisting of two G1312A binary pumps, an in-line G1379A degasser, an G1329A autosampler, a G1316A column oven and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (100 mm x 3.0 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase consisting of a 2:98 (v/v) mixture of acetonitrile

and 0.2% (v/v) formic acid in water at 40 ºC with a flow rate of 0.8 mL/min. The detection of methyldopa was performed in multiple reaction monitoring (MRM) mode using an ion trap mass spectrometer equipped with an electrospray ion (ESI) source, positive ionisation (capillary 4500 V, nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, dry gas temperature 350ºC). The extracted ion current (EIC) chromatogram of m/z (139.2, 166.2, 195.2) from m/z 212 was analyzed.

Standard solutions

A stock solution (4.0 mg/mL) was prepared by dissolving an appropriate quantity of methyldopa in methanol. A working solution (32 µg/mL) was prepared by appropriate dilution in drug-free human plasma. This solution was used to prepare plasma calibration standards with the concentrations of 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, and 20.48 µg/mL. Quality control (QC) samples of 0.64 µg/mL (low), 2.56 µg/mL (medium) and 10.24 µg/mL (high) were prepared by adding the appropriate volumes of working solution to drug-free human plasma. The obtained plasma calibration standards and quality control standards were pipetted into 15 mL polypropylene tubes and stored at -20ºC until analysis.

Sample preparation

Standards and plasma samples (0.2 mL) were deproteinised with methanol (0.6 mL). After vortex-mixture (10 s) and centrifugation (2 min at 12000 rpm), the supernatants (0.2 mL) were transferred to autosampler vials, and 1.5 µL were injected into the HPLC system.

Method validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the plasma samples containing methyldopa with those obtained from different plasma blank samples (n=6).

The concentration of methyldopa was determined automatically by the instrument data system using peak areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = c + bx + ax^2$, weighted (1/y) quadratic regression, where y - peak area of the analyte and x - concentration of the analyte (µg/mL).

The intra-day precision (expressed as coefficient of variation, CV %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %) were determined by analysing five different samples (n = 5) from each QC standards (at low, medium and high levels) on the same day. The inter-day precision and accuracy were determined by analysing one sample from each of the QC standards (at low, medium and high levels), in the course of five different days (n = 5).

The lower limit of quantification (LLOQ) was established as the lowest calibration standard with an accuracy and precision less than 20%.

The relative recoveries (at LLOQ, low, medium and high levels) were measured by comparing the response of the spiked plasma with the response of standards in solvent with the same concentration of methyldopa as the plasma (n = 5).

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