

# Liquid Chromatography Tandem Mass Spectrometry Simultaneous Determination of Amlodipine and Telmisartan in Human Plasma for Therapeutic Drug Monitoring

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*A rapid high-throughput liquid chromatography method with tandem mass spectrometry detection (LC-MS/MS) was developed for therapeutic drug monitoring of amlodipine and valsartan. A Zorbax SB-C18 column was used for separation with mobile phase composition of 0.1% formic acid in water and acetonitril in gradient elution, with a flow rate of 1 mL/min. Detection was performed in MS/MS mode (sum of m/z 238.2 and 294.2 from m/z 410.2 for amlodipine and m/z 497.4 from m/z 515.4 for telmisartan) using heated electrospray positive ionization. Valsartan was used as internal standard. Single-step protein precipitation with methanol was used for the preparation of plasma samples. The method was validated with regards to selectivity, linearity and accuracy over a range of 1 - 64 ng/mL plasma for amlodipine and 11.5 - 736 ng/mL plasma for telmisartan. The analytical method is rapid, simple, selective and sensitive and is suitable for use in bioanalysis and therapeutic drug monitoring.*

*Keywords: amlodipine, telmisartan, LC/MS, human plasma*

Recognized as a global public health issue, despite of an increasing trend in awareness and available treatment strategies, hypertension requires a careful and individualized therapeutic approach. [1] Prevalence of hypertension in Romanian is 40.41%, awareness of hypertension is 69.55%, with 59.15% hypertensive individuals under current treatment with a control rate of 25%. In the last 7 years, there has been a 10.7% decrease in hypertension's prevalence together with an increase by 57% in awareness of hypertension and an increase by 52% in treatment of hypertension, leading to almost doubling of the hypertension's control rate in all hypertensive individuals. [2] Using complementary mechanisms, associations of antihypertensive drugs as a single-pill medication, proved to be an effective, safer and adherence-increasing solution for blood pressure control. [3]

Telmisartan/ amlodipine as a single-pill combination available in several strengths, demonstrated superior efficacy to either monotherapy, in various patient populations, including those with diabetes, obesity or renal disease. [4] Although comorbidities, concomitant therapies and complex individual particularities are also important, the hypotensive effects of medications are generally dependent on the evolution of the plasma drug concentration-time profile. [5] Therefore, the parameters obtained from the analysis of the potential relationship between plasmatic drug concentrations and hypotensive effects could be used to predict the evolution of the blood pressure response to an available range of doses, allowing for therapy individualization and optimization. [6] Furthermore, therapeutic drug monitoring has been suggested as a reliable and cost-effective method of blood

pressure control among patients diagnosed with resistant hypertension. [7] The latter was frequently associated with patient's non-adherence to the prescribed antihypertensive regimen and, in this context, drugs' or active metabolites' plasmatic concentrations could represent an objective and practical method for adherence evaluation. [8]

The objective of this study was to develop a fast method for the determination of amlodipine and telmisartan plasma concentrations for therapeutic drug monitoring. Mass spectrometry is a very versatile and sensitive detection technique, as mass spectrometry can be coupled with either gas chromatographic separation methods or liquid chromatographic separation methods and can be used for a wide array of determinations such as identification of PDE-5 inhibitors in food supplements [9], identification and quantitation of pesticides in soil [10], identification of psychoactive substances in ethnobotanical products [11] or determination of acrylamide in bread [12]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is widely used for the determination of drug plasma levels due to being precise, accurate, sensitive and robust.

Currently there is a large number of methods described in literature for the determination of amlodipine and telmisartan in human plasma. Amlodipine has been determined with a wide array of methods, such as reverse phase high performance liquid chromatography (RP-HPLC) [13], high performance liquid chromatography with fluorescence detection [14] or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [15,16]. Sample processing methods widely used for amlodipine samples are LLE (LLE - liquid/liquid extraction)

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[16] and SPE (SPE - solid phase extraction) [14,15] however these techniques are tedious and costly. Telmisartan has also been quantified from human plasma by various methods, such as performance liquid chromatography (HPLC) with fluorescence detection [17] or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [18]. Liquid-liquid extraction has often been used for sample preparation of telmisartan plasma samples [17, 18].

The aim in developing our LC-MS/MS method was obtaining optimal parameters regarding sample throughput (both sample preparation and chromatographic analysis) in order to use this method for rapid analysis of samples in short time, as needed in therapeutic drug monitoring.

## Experimental part

### Reagents

Gradient grade methanol and acetonitril, as well as formic acid of analytical purity were manufactured by Merck KGaA (Darmstadt, Germany). Bidistilled deionized water was used. Human blank plasma was supplied by the Regional Blood Transfusion Center Cluj-Napoca (Romania) and was obtained from the healthy volunteers.

### Apparatus

Chromatographic separation was performed on an Agilent Technologies (Santa Clara, USA) 1100 HPLC system consisting of an G1316A Column Thermostat, G1329A Autosampler, G1312A Binary Pump, G1379A Degasser coupled with an Agilent Ion Trap Detector (1100, SL). Other equipment used were: Sigma Laborzentrifugen GmbH (Osterode, Germany) 204 centrifuge; Mettler-Toledo (Greifensee, Switzerland) Analytical Plus balance; a Scientific Industries Inc. (New York, USA) Vortex Genie 2 vortex mixer; Biohit (Helsinki, Finland) Proline series automatic pipettes.

### Liquid chromatography tandem mass spectrometry conditions

The analytical separation of amlodipine, telmisartan and internal standard (IS), valsartan, was achieved on a Zorbax SB-C18 3.0 x 100mm, 3.5  $\mu$ m chromatographic column (Agilent Technologies) thermostatted at 40°C with a mobile phase consisting of acetonitrile and 0.1% formic acid in water in gradient elution with the following program: 37% acetonitril from 0.0 to 1.1 min., 55% acetonitril from 1.1 to 2.5 min, 37% acetonitril from 2.5 to 3.5 min. Total runtime was 3.5 minutes and flow rate was 1 mL/min. Detection of amlodipine, telmisartan and valsartan was performed using MS/MS mode, monitoring the transitions: sum of  $m/z$  238.2 and  $m/z$  294.2 from  $m/z$  410.2 for amlodipine,  $m/z$  497.4 from  $m/z$  515.4 for telmisartan and sum of  $m/z$  306.2 and  $m/z$  362.2 from  $m/z$  436.5 for valsartan; using positive electrospray ionization source.

### Standard solutions

Pharmaceutical purity reference standards of amlodipine, telmisartan and valsartan were used. Stock solutions of amlodipine and telmisartan in methanol were further diluted with plasma to obtain intermediary solutions. These two solutions were used to obtain the calibration working solutions. A stock solution with a concentration of 25 ng/mL valsartan in methanol was prepared to be used as internal standard solution. The working solutions along with the internal standard solution were then used to prepare plasma calibration standards with concentrations of 1 (lower limit of quantification - LLOQ), 2, 4, 8, 16, 32 and 64 ng/mL for amlodipine and 11.5 (lower

limit of quantification - LLOQ), 13, 46, 92, 184, 368 and 736 ng/mL for telmisartan.

### Pre-treatment of samples

Plasma working solutions and plasma volunteer samples (200  $\mu$ L) were spiked with 25ng/mL internal standard solution (100  $\mu$ L) and deproteinized with methanol (500  $\mu$ L) in Eppendorf tubes, mixed with the vortex mixer (10 seconds) and finally centrifuged (5 min at 12000 rpm). Volumes of 300  $\mu$ L of the obtained supernatant were transferred to chromatographic vials and inserted into the auto-sampler. Volumes of 10  $\mu$ L of sample were injected into the LC-MS system.

### Method validation

An evaluation of selectivity was carried out for the analytical method. To prove selectivity the chromatograms of plasma samples spiked with fluconazole were compared to the ones obtained from blank plasma samples. Chromatograms were processed using QuantAnalysis (Brucker Daltonics, Germania) software. Concentrations were calculated automatically by the instrument data systems with a processing method using the internal standard method. Calibration curves were linear, constructed from single calibration standards, the weighting factor was  $1/y^2$ .

Five series of calibration standards for both amlodipine and telmisartan were analysed on the same day to determine intra-day precision (expressed as coefficient of variation, CV, %) and accuracy (expressed as relative difference between obtained and theoretical concentration, bias %). Five series of calibration standards for both amlodipine and telmisartan, each series in a different run, on different days, were also analysed in order to establish inter-day accuracy and precision.

The lowest calibration standards for amlodipine and telmisartan were also extensively studied. Five different samples of LLOQ at the concentration levels of 1 ng/mL and 11.5 ng/mL telmisartan were analysed for calculating both intra- and inter-day accuracy and precision.

## Results and discussions

For the studied analytes it was concluded that positive electrospray ionization (ESI) mode gave better spectrometer responses than the negative mode and the atmospheric pressure chemical ionization (APCI) mode. Amlodipine forms a pseudomolecular ion  $[M+H]^+$  ( $m/z$  410.2) by accepting a proton in the acidic mobile phase and was fragmented to  $m/z$  238.2 and  $m/z$  294.2. Telmisartan also forms a pseudomolecular ion  $[M+H]^+$  ( $m/z$  515.4) by accepting a proton in the acidic mobile phase and was fragmented to  $m/z$  497.4. The fragments  $m/z$  306.2 and  $m/z$  362.2 fragmented from  $m/z$  436.5 were monitored for the internal standard, valmisartan.

To achieve good chromatographic peaks with minimal tailing and good resolution the developed liquid chromatography tandem mass spectrometry (LC-MS/MS) method was optimized. Best results were obtained using a Zorbax SB-C18 3.0 x 100mm, 3.5 $\mu$ m column thermostatted at 40°C and a mixture of acetonitril and 0.1% formic acid in water mobile phase under gradient conditions with a flow rate of 1 mL/min. Analysis of blank samples showed no interfering endogenous peaks at the retention times of amlodipine (1.45 min), telmisartan (1.95 min) and valmisartan (3.05 min) in human plasma (fig. 1).

The method was linear for both amlodipine and telmisartan with calibration curves having correlation coefficients higher than 0.991 throughout the whole range

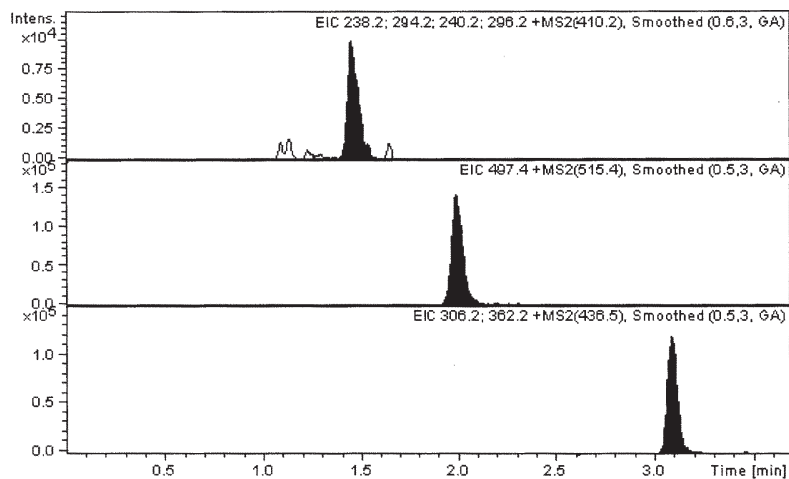


Fig. 1. Chromatograms of amlodipine (upper figure, 1ng/mL), telmisartan (middle figure, 11.5ng/mL) and valsartan (lower figure, 25ng/mL) peaks for standard solutions at lower limit of quantification.

**Table 1**  
WITHIN-RUN ACCURACY, PRECISION AND RECOVERY FOR  
AMLODIPINE

$c_{\text{nominal}}$ (ng/ml)	Mean $c_{\text{found}}$ (ng/ml ( $\pm$ SD))	Bias (%)	CV (%)	Mean recovery (% ( $\pm$ SD))
1.000	1.073 (0.100)	7.3	9.3	91.6 (5.3)
2.000	2.010 (0.114)	0.5	5.7	93.4 (5.6)
8.000	7.699 (0.447)	-3.8	5.8	93.8 (5.2)
32.000	32.931 (1.239)	2.9	3.8	91.8 (8.0)

**Table 3**  
WITHIN-RUN ACCURACY, PRECISION AND RECOVERY FOR  
TELMISARTAN

$c_{\text{nominal}}$ (ng/ml)	Mean $c_{\text{found}}$ (ng/ml ( $\pm$ SD))	Bias (%)	CV (%)	Mean recovery (% ( $\pm$ SD))
11.50	12.40 (1.64)	7.8	13.2	99.6 (5.8)
23.00	21.00 (1.00)	-8.7	4.8	97.1 (5.9)
92.00	91.00 (3.61)	-1.1	4.0	104.2 (5.8)
368.00	361.33 (18.72)	-1.8	5.2	91.8 (8.0)

**Table 2**  
BETWEEN RUN ACCURACY, PRECISION AND RECOVERY FOR  
AMLODIPINE

$c_{\text{nominal}}$ (ng/ml)	Mean $c_{\text{found}}$ (ng/ml ( $\pm$ SD))	Bias (%)	CV (%)	Mean recovery (% ( $\pm$ SD))
1.000	1.066 (0.111)	6.6	10.4	99.0 (5.2)
2.000	2.128 (0.170)	6.4	8.0	95.1 (3.8)
8.000	7.916 (0.864)	-1.0	10.9	97.0 (5.4)
32.000	31.953 (2.609)	-0.1	8.2	95.9 (9.6)

**Table 4**  
BETWEEN RUN ACCURACY, PRECISION AND RECOVERY FOR  
TELMISARTAN

$c_{\text{nominal}}$ (ng/ml)	Mean $c_{\text{found}}$ (ng/ml ( $\pm$ SD))	Bias (%)	CV (%)	Mean recovery (% ( $\pm$ SD))
11.50	11.19 (1.34)	-2.7	11.9	103.4 (4.7)
23.00	22.43 (2.05)	-2.5	9.1	92.2 (5.8)
92.00	95.10 (6.15)	3.4	6.5	99.5 (1.5)
368.00	359.14 (30.57)	-2.4	8.5	96.5 (5.2)

of concentrations studied (1- 64 ng/mL for amlodipine and 11.5 - 736 ng/mL for telmisartan). Values obtained for within run and between run precision, accuracy and recovery during method validation are shown in table 1 and table 2 for amlodipine and table 3 and table 4 for telmisartan.

There are few methods described for the determination of amlodipine and telmisartan in plasma by RP-HPLC, HPLC with fluorescence detection or LC-MS/MS, alone, combined or simultaneously with other analytes [13- 18]. Brinker et al. developed and validated a RP-HPLC method for determining amlodipine from human plasma. This method has the disadvantage of longer runtimes and less sensitivity in comparison to LC-MS/MS methods [13].

Tatar et al. developed a high performance liquid chromatography (HPLC) method with fluorescence detection for the determination of amlodipine from human plasma. While this method is very sensitive, it requires a

laborious, time consuming and expensive sample preparation which involves derivatization with 4-chloro-7-nitrobenzofurazan, solid-phase extraction on a silica column [14].

Hemanth et al. developed an LC-MS/MS method using solid phase extraction of amlodipine from human plasma as sample preparation technique. Solid phase extraction is time consuming and involves higher costs in comparison to the protein precipitation technique used by us. The longer runtime can also prove to be a disadvantage when a large number of samples need to be analyzed [15].

Chan-Mei et al. developed a method for the determination of amlodipine using liquid chromatography coupled with tandem mass spectrometry. Liquid-liquid extraction was used for sample preparation, a technique which is not only costly but also very time consuming. The total runtime of the method was 10 min, considerably

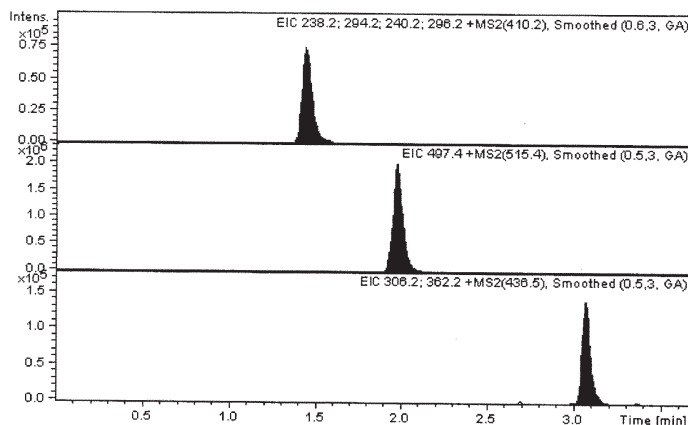


Fig. 2. Typical chromatogram of amlodipine (upper figure, concentration found 14.49ng/mL), telmisartan (middle figure, concentration found 196.2ng/mL) and valsartan (lower figure) after oral administration of telmisartan/amlodipine 40/5 mg dose to a subject

longer than the runtime of the method developed by us [16].

Shen et al. developed an HPLC method for determination of telmisartan plasma concentrations with applicability in pharmacokinetic studies. Samples were processed using liquid-liquid extraction. While their method proved to have applicability in pharmacokinetic studies, the method developed by our laboratory had the advantage of easier and shorter sample processing, and better selectivity due to the use of tandem mass spectrometry [17].

Yan et al. developed a method using liquid chromatography coupled with tandem mass spectrometry for the determination of telmisartan with samples being processed using liquid-liquid extraction. While the method had good sensitivity the longer sample processing times and higher costs due to the extraction technique are a disadvantage in comparison with the method developed by us which where samples are being processed by protein precipitation, a much easier, faster and cheaper technique [18].

The method developed and validated is simple, selective and sensitive, requires a small plasma sample volume (200  $\mu$ L) and requires a sample preparation technique which is simple and inexpensive.

Results proved a good linearity of the calibration curves, sensitivity (LLOQ of 1 ng/mL for amlodipine and 11.5 ng/mL for telmisartan), as well as accuracy and precision over the entire concentration ranges studied. Accuracy and precision were within limits provided by international guidelines [19, 20].

The objective of the study was to develop and validate a rapid, simple, selective, sensitive high throughput LC-MS/MS method for the determination of both amlodipine and telmisartan in human plasma samples which could be applied in clinical drug monitoring, bioavailability-bioequivalence studies or other pharmacokinetic studies (e.g. drug-drug interaction studies). A typical plasma sample of amlodipine and telmisartan obtained from a patient after single administration of telmisartan/amlodipine in doses of 40mg/5mg is shown in figure 2.

## Conclusions

The LC-MS/MS method developed and validated by us stands out through simplicity, sensitivity, selectivity and accessibility. In contrast with other methods reported in scientific literature for quantification of amlodipine and telmisartan in plasma [13-18] this method is fast, robust and less expensive, important characteristic for a high-throughput method which needs to be used in routine analysis. It can be applied in clinical and bioequivalence studies, clinical level monitoring and pharmacokinetics.

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