New high-throughput liquid chromatographic tandem mass spectrometry assay for therapeutic drug monitoring of carvedilol in children with congestive heart failure

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Abstract

A new high-throughput liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for the quantification of carvedilol in human plasma was developed and validated. The separation was performed on a Zorbax SB-C18 column under isocratic conditions using a 34:66 (v/v) mixture of acetonitrile and 0.2% (v/v) formic acid in water at 42 ºC with a flow rate of 0.3 mL/min. The detection of carvedilol was performed in multiple reaction monitoring (MRM) mode (m/z 222, 224, 283 from m/z 407). The human plasma samples (0.2 mL) were deproteinized with methanol, and aliquots of 15 µL from the supernatants obtained after centrifugation were directly injected into the chromatographic system. The method shows a good linearity (r > 0.9979), precision (CV < 12.9 %) and accuracy (bias < 7.8 %) over the range of 1.63-180.9 ng/mL plasma. The lower limit of quantification (LLOQ) was 1.63 ng/mL and the recovery was between 95-109.8 %. The method is not expensive, it needs a minimum time for plasma sample preparation and has a run-time of 2 min for instrument analysis (retention time of carvedilol was 1.75 min). The developed and validated high-throughput method is very simple, rapid and efficient, with wide applications in clinical therapeutic drug monitoring, pharmacokinetics and bioequivalence studies.

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

Rezumat

S-a elaborat şi s-a validat o nouă metodă de cromatografie de lichide de înaltă performanţă cuplată cu spectrometrie de masă în tandem (LC-MS/MS) pentru dozarea carvedilolului în plasma umană. Separarea s-a realizat pe o coloană Zorbax SB-C18 în condiţii izocratice folosind un amestec de acetonitril şi 0.2% (v/v) acid

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formic în apă (34:66, v/v) la 42 ºC cu un debit de 0.3 mL/min. Detecţia carvedilolului s-a realizat prin monitorizarea reacţiilor multiple (MRM) (m/z 222, 224, 283 din m/z 407). Probele de plasmă umană (0.2 mL) s-au deproteinizat cu metanol şi din supernatantul obţinut după centrifugare s-au injectat direct în cromatograf un volum de 15 μL. Metoda prezintă o bună linearitate (r > 0.9979), precizie (CV < 12.9 %) şi acurateţe (bias < 7.8 %) pe domeniul de concentraţii 1.63-180.9 ng/mL plasmă. Cea mai mică limită de cuantificare (LLOQ) a fost de 1.63 ng/mL şi randamentul de recuperare din plasmă a fost cuprins între 95-109.8 %. Metoda nu este costisitoare, necesită un timp minim pentru pregătirea probelor de plasmă şi are un timp de analiză de 2 min pentru determinarea instrumentală (timpul de retenţie al carvedilolului a fost de 1.75 min). Metoda elaborată şi validată este foarte simplă, rapidă şi eficientă, având largi aplicaţii în monitorizarea carvedilolului în timpul tratamentelor clinice, în studiile de farmacocinetică şi bioechivalenţă.

Cuvinte cheie: *carvedilol, LC-MS/MS, monitorizarea medicamentelor în terapie*

Introduction

Carvedilol (CVD; *1-(9 H- Carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy-)ethyl]-amino]-2-propanol), Figure 1*, is a noncardioselective β/α$_1$-adrenoreceptor blocker and has an anti-oxidant effect. It is used as an antihypertensive and an antiangina drug, being recognized as an effective agent for the treatment of congestive heart failure (CHF) (1). It is administered as a racemic mixture, although enantiomers exhibit different pharmacological effects: both enantiomers are equally potent α$_1$-adrenoreceptor antagonists, but the β-blockade is exerted mainly by S(-)-CVD.

The pharmacokinetics of CVD is stereoselective. CVD is well absorbed after oral administration but its absolute bioavailability is low, about 25–30% (15% for S(-)-CVD and 31% for R(+)-CVD) due to an extensive first-pass metabolism. The concentration peak in plasma occurs after about 1-2 h and the plasma levels are linearly related to the administered dose, up to 0,3 mg/L. CVD is extensively bound (≥ 95%) to plasma proteins, R(+)-enantiomer being more tightly bound. The half-life

of CVD in plasma is of 4-8 hours. Only 1% of CVD is excreted unchanged in urine (2, 3).

CVD is more frequently used in adult therapy whereas, in pediatrics, there are very few studies which led to disputed conclusions (4-6). In 2001, the first two articles regarding CVD's pediatric use were published, followed, in the next years by only a few more (7-11). The pharmacokinetics research on CVD in pediatrics is scant. Laer et al. reported a study in which the traditional therapy failed in the case of the 15 children with CHF (aged between 6 weeks and 19 years) observed (12). They compared CVD's pharmacokinetics in the case of those 15 children with the one found in the case of 9 healthy adults. The diminishing to half time was significantly shorter at children in comparison with adults (2.9 in contrast with 5.2 hours). By dividing the children in two groups (under and above 3.5 years old), the medium time registered was 2.2 hours at children under 3.5 years and 3.6 hours in the case of those above the mentioned age. Because of the interest in the pediatric therapy with CVD, this medicine was included on the list of off-patent medicinal products for pediatric studies (13) published by European Medicines Agency in 2007.

Several methods involving high-performance liquid-chromatography (HPLC) with fluorescence detection (14-19), electrochemical detection (20) or mass spectrometry detector (21, 22) and capillary electrophoresis (CE) (23,
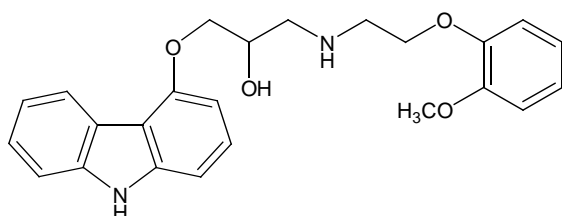

Figure 1. Chemical structure of carvedilol

24) have been reported to determine therapeutic levels of CVD in plasma.

CVD is mainly detected by fluorescence measurement. Because it is strongly bound to proteins, most methods involve primarily precipitation of proteins (14, 17) and/or extraction of CVD in organic solvents. Generally, CVD is isolated by liquid-liquid extraction (LLE) (15, 20, 21, 25) or solid-phase extraction (SPE) (16, 24), a time-consuming step that increases the cost of assay and can affect the recovery. An appropriate derivatisation (19, 23, 26) or the usage of a chiral column (18, 25) is necessary for the determination of CVD enantiomers, otherwise the method is more expensive and the run-time is longer.

Recently, the liquid chromatography tandem mass spectrometry (LC-MS/MS) assay offers considerable advantages by its powerful performances: speed, selectivity, sensitivity and robustness. Sample preparation is more simple and rapid and often includes precipitation of proteins (PP) and derivatisation (for chiral analysis) (22, 26) or LLE (21, 27, 28) before chromatographic analysis.

The aim of this work was to develop and validate a new simple and efficiently high throughput LC-MS/MS assay for quantification of CVD in human plasma in clinical level monitoring.

Material and methods

Reagents

Carvedilol (CVD) was reference standard from Sigma (St. Louis, MO, USA). Acetonitrile of HPLC-grade and methanol and 98% formic acid of analytical-reagent grade were purchased from Merck KGaA (Darmstadt, Germany). Bidistilled, deionized water pro injectiones was purchased from Infusion Solution Laboratory of University of Medicine and Pharmacy Cluj-Napoca (Romania). The human blank plasma was supplied by the Transfusion Center Cluj-Napoca (Romania) from the healthy volunteers, men and women.

Apparatus

The following apparatus were used: 204 Sigma Centrifuge (Osterode am Harz, Germany); Analytical Plus 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 a G1312A binary pump, an in-line G1379A degasser, an G1329A autosampler, a G1316A column thermostat and an Agilent Ion Trap Detector 1100 VL.

Chromatographic and spectrometric conditions

Chromatographic separation was performed on a Zorbax SB-C18 (50 mm x 2.1 mm i.d., 3.5 μm) column (Agilent Technologies) under isocratic conditions using a mobile phase of a 34:66 (v/v) mixture of acetonitrile and 0.2% (v/v) formic acid in water at 42ºC and a flow rate of 0.3 mL/min. The detection of CVD was in the multiple reaction monitoring (MRM) mode (monitored ions: m/z 222, 224, 283 from m/z 407) using an ion trap mass spectrometer equipped with an atmospheric pressure electrospray ionization ion source (polarity: positive, nebulizer 40 psi (nitrogen), dry gas nitrogen at 9 L/min, dry gas temperature 350ºC, ICC Target: 50000, Charge control: ON). The run-time was of 2 minutes.

Standard solutions

A stock solution of CVD (1,005 mg/mL) was prepared by dissolving an appropriate quantity of CVD in methanol. A working solution (12,060 μg/mL) was prepared by appropriate dilution in methanol and another one (189,9 μg/mL) by appropriate dilution in drug-free human plasma. These solutions were used to prepare plasma standards with the concentrations of 1.63, 3.62, 7.24, 14.47, 28.94, 57.89, 86.83 and 180.90 ng/mL. Quality control (QC) samples of 5.43 ng/mL (lower), 27.14 ng/mL
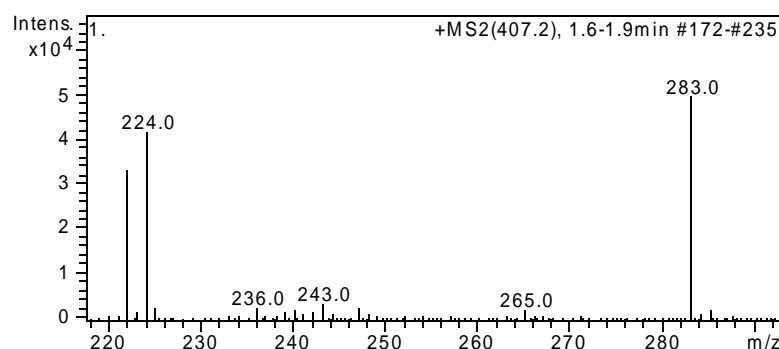
Figure 2. MS/MS spectra of carvedilol

(medium) and 108.54 ng/mL (higher) were prepared by adding the appropriate volumes of working solution to drug-free human plasma. The resultant plasma standards and quality control standards were pipetted into 15 mL polypropylene tubes and stored -20°C until analysis.

For sample dilution validation, two dilutions will be freshly prepared on the day of analysis (482.4 and 48.24 ng/mL) by adding the appropriate volumes of working solution to drug-free human plasma.

Sample preparation

Standards and plasma samples (0.2 mL) were deproteinized with methanol (0.6 mL). After vortex-mixture (10 s) and centrifugation (6 min at 6000 rpm), the supernatants were transferred in autosampler vials and 15 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 CVD with those obtained from different plasma blank samples (n=6).

The concentration of CVD was determined automatically by the instrument data system using pick areas and the external standard method. The calibration curve model was determined by the least squares analysis: $y = b + ax$, weighted ($1/y$) linear regression, where y - peak area and x - analyte concentration (ng/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 the analysis on the same day of five different samples (n = 5) from each QC standards (at lower, medium and higher levels). The inter-day precision and accuracy were determined by the analysis on five different days (n = 5) of one sample from each QC standards (at lower, medium and higher levels).

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

The absolute recoveries were measured by comparing the response of CVD from spiked plasma samples with the response of CVD from a standard solution with the same analyte concentration, prepared in mobile phase and processed in the same manner with plasma sample. The absolute recovery was evaluated for quantification limit and also for each QC level.

The CVD stability in plasma at lower and higher levels (n=5) was investigated. For the post-preparative stability (PPS) study, the samples were prepared and analyzed immediately and after 10 h (kept at 25°C), and all were calculated against the same calibration curve. For the long-term stability (LTS) study, the samples were stored below -20°C and analyzed during 2 months. The concentrations of the stored samples were calculated against calibra-
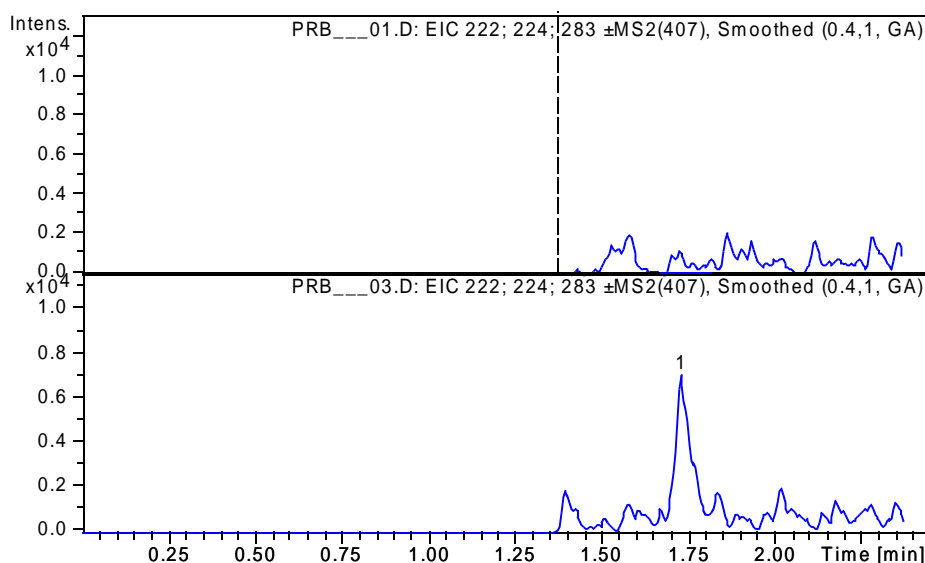
Figure 3. Representative chromatograms of (up) blank plasma and (down) plasma spiked with carvedilol at lower limit of quantification (1.63 ng/mL) (RT=1.75 min)

tion curve of the day and the mean values were compared with the nominal concentrations. For the freeze-thaw stability (FTS) study, the samples were submitted to three freeze-thaw cycles in three consecutive days. After the third cycle the samples were analyzed against the calibration curve of the day and the mean values of concentrations were compared with the nominal concentrations. The requirement for the stability of drug is that the difference between mean concentrations of the tested samples in various conditions and nominal concentrations should be in ±15% range.

Results

The chromatographic conditions, especially the composition of the mobile phase, were optimized in several trials to achieve a good MS signal, a short retention time of CVD and consequently a high-throughput analysis. The best results were obtained with the mixture of acetonitrile and 0.2% (v/v) formic acid in water (34:66, v/v) under isocratic conditions. In the selected chromatographic conditions the retention time of CVD was 1.63 min and the ana-

lytical run-time was 2 min. The detection of CVD was performed in MRM mode by monitoring ions with m/z 222, 224 and 283 obtained from parent ion of CVD with m/z 407 (*Figure 2*).

Representative chromatograms of drug-free plasma and plasma spiked with CVD at LLOQ are shown in *Figure 3*. No interfering peaks from the endogenous plasma components were observed in the retention time of CVD.

The calibration curves were linear over the concentration range of 1.63 – 180.9 μg/mL in human plasma, with a correlation coefficient greater than 0.9979. The LLOQ was 1.63 μg/mL. The values obtained for intra-day and inter-day precision and accuracy during the validation for plasma are shown in *Table 1* and *Table 2*, respectively.

Discussion

We propose a very simple and rapid pretreatment of plasma samples including only PP by methanol and direct injection into chromatographic system from the supernatant obtained after centrifugation.

Table 1. The intra-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of carvedilol in human plasma (n = 5)

Nominal concentration (ng/mL)	Found concentration mean		CV (%)	Bias (%)	Recovery	
	ng/mL	± SD			(%)	± SD
1.63	1.76	0.11	6.5	7.8	107.6	6.2
5.43	5.70	12.9	12.9	5.0	109.8	15.8
27.14	28.18	4.5	4.5	3.9	108.6	4.9
108.54	111.25	3.0	3.0	2.5	104.8	3.2

Table 2. The inter-day precision (CV %) and accuracy (bias %) and recovery data for the measurement of carvedilol in human plasma (n = 5)

Nominal concentration (ng/mL)	Found concentration mean		CV (%)	Bias (%)	Recovery	
	ng/mL	± SD			(%)	± SD
1.63	1.71	0.12	6.8	4.8	101.4	4.1
5.43	5.27	0.54	10.3	-2.9	95.0	13.7
27.14	28.14	1.18	4.2	3.7	106.4	8.0
108.54	108.19	4.97	4.6	-0.3	96.9	4.4

All the other methods LC-MS/MS reported in the literature for the quantification of carvedilol in plasma include an isolation step by LLE (21, 25, 27, 28) to eliminate the impurities and to increase the sensitivity. But this operation increases the time of analysis, increases the costs and can affect the recovery. Therefore, do Carmo Borges et al. (21) and Jeong et al. (28) obtained a LLOQ of 0,1 ng/mL after extraction of carvedilol with diethyl-ether and tert-butyl ether, respectively, but the recoveries were only between 80.8 - 83.9% and 79.1 - 84.1%, respectively. In our method, the sample preparation is very simple and rapid and offers a shorter time of analyses and a lower cost in comparison with the other longer methods which used extraction prior to LC assays, with better recoveries (95-109.8 %).

Zarghi et al have also analyzed CVD from human plasma after only PP with acetonitrile. For quantification they used a HPLC method with fluorescence detection and obtained a LLOQ of 1 ng/mL with an average of recovery of 98.1 ± 2.2%. However, the run-time of analytical method was 5 min, too long for a high-throughput determination (17).

LC-MS/MS assay

Our method is very simple and rapid, the analytical run-time being of 2 min. Do Carmo Borges et al. have also obtained a good run-time of 3.5 min and a retention time of CVD of 1.6 min, but after LLE of CVD from plasma matrix (21). The method reported by Jeong et al. is a high-throughput method too, with a run-time of 2.5 min and a retention time of CVD of 1.44 min, but they also have included a LLE step prior LC-MS/MS assay (28), which increases considerably the total analysis time.

Assay validation

The method was validated in accordance with international regulations (29, 30, 31). The obtained results proved a good linearity (r > 0.9979), sensitivity (LLOQ - 1.63 µg/mL), accuracy and precision over the studied concentration range. All values for accuracy and precision were within recommended limits (*Table 1* and *Table 2*). The recovery values were between 95-109.8 %, which means no analyte loss during sample preparation due to adsorption on precipitated proteins.
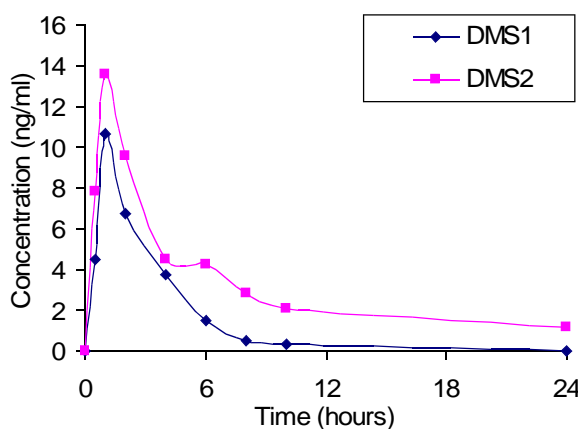
Figure 4. Carvedilol plasma levels on a subject (code DMS) after administration of carvedilol 0.1 mg/kg (profile DMS1) and 0.2 mg/Kg (profile DMS2), respectively

Plasma dilutions could be made with a CV% less than 6.3% and accuracy less than 4.9% for within-run and between-run determinations.

CVD showed good post-preparative stability in autosampler for at least 10 hours at 25°C before the chromatographic assay (CV of 4.4% at lower level and 1.4% at higher level, respectively), a good long-term stability in plasma stored below -20°C for 2 months (CV of 6.8% at lower level and 0.5% at higher level, respectively) and a good freeze-thaw stability in plasma submitted of three freeze-thaw cycles (CV of 6.9% at lower level and 9.7% at higher level, respectively).

Our developed LC-MS/MS assay is simple, rapid, accurate and not expensive. In comparison with other published LC-MS/MS assays (21, 25, 27, 28) for CVD level monitoring in plasma our method performs better in terms of speed (both sample preparation and chromatographic run-time) and costs, which are essential attributes for methods used in routine analysis. The method was validated over the concentration range of 1.63-180.9 ng/mL and was validated for the sample dilution too, being assured the covering of the therapeutic plasma levels of CVD (<300 ng/mL (3)). This high-throughput method can be successfully applied in clinical level monitoring of CVD and it can have wide applications in pharmacokinetics and bioequivalence studies too.

Method application

The developed and validated analytical method was used for therapeutic drug monitoring and pharmacokinetic parameter estimation in children with CHF. A typical plasma profile of carvedilol obtained form a patient (code: DMS, 15 months, weight 8.25 Kg, diagnostic: congestive heart failure NYHA III), after administration of two doses of carvedilol (0.1 and 0.2 mg/kg) is showed in *Figure 4*.

Acknowledgement

Abbreviations

ACEI - angiotensin conversion enzyme inhibitors
CE - capillary electrophoresis
CHD - congenital heart diseases
CHF - congestive heart failure
CVD - carvedilol
FTS - freeze-thaw stability
HPLC - high-performance liquid-chromatography
LC-MS/MS - liquid chromatography tandem mass spectrometry
LLE - liquid-liquid extraction
LLOQ - lower limit of quantification
LTS - long-term stability
MRM - multiple reaction monitoring
PP - precipitation of proteins
PPS - post-preparative stability
QC - quality control
SPE – solid-phase extraction

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