Determination of Carvedilol in Human Plasma by Gas Chromatography–Mass Spectrometry Method

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Abstract

An analytical procedure was developed and validated for the quantitation of carvedilol in human plasma. Carvedilol and atenolol (internal standard) were extracted from human plasma with a mixture of diethylether and ethylacetate at basic pH with liquid–liquid extraction. The extracts were derivatized with *n*-Methyl-*n*-(trimethylsilyl)trifluoroacetamide and analyzed by gas chromatography-mass spectrometry (GC–MS). Calibration curves were linear over the concentration range 15–500 ng/mL. Intra- and inter-day precision, expressed as the relative standard deviation were less than 8.0%, and accuracy (relative error) was better than 11.0%. The limits of detection and quantification of carvedilol were 5.0 and 15 ng/mL, respectively. Also, the method was successfully applied to three patients with hypertension who had been given an oral tablet of 25 mg carvedilol.

Introduction

Carvedilol, (±)-1-(carbazol–4-yloxy)-3-((2-(*o*-methoxyphenoxy)ethyl)amino)-2-propanol, is a nonselective β-blocking agent. It also has vasodilating properties that are attributed mainly to its blocking activity at α_1-receptors. Carvedilol is a racemic compound, and the nonselective β-blocking activity resides mainly in the (*S*)-carvedilol, while the α-blocking activity is shared by (*R*)- and (*S*)-enantiomers (1,2). But carvedilol is used clinically as a racemic mixture of both enantiomers. It is used in the treatment of mild to moderate hypertension and angina pectoris (3) and is often used in combination with other drugs.

Several methods have been reported for determination of carvedilol including high-performance liquid chromatography (HPLC) (4–9) and liquid chromatograph–tandem mass spectrometry (LC–MS–MS) (10–12).

-Blockers have similar chemical structures with highly polar functional groups that yield them unsuitable for analysis by gas chromatography (GC) methods. Recently the use of mass selective detectors with a capillary GC coupled to MS as a mode of detection has considerably increased. Suitable derivatization should improve the gas chromatographic properties of the compounds and yield compounds with mass spectra containing high relative intensity and high-mass fragments suitable for selected ion monitoring (SIM) (13).

In addition, at the date of submission no method has been reported for determination of carvedilol by GC–MS from human plasma. Therefore, a GC–MS with SIM method for determination of carvedilol after a derivatization procedure in human plasma using internal standard methodology is reported.

The developed method was validated by using linearity, stability, precision, accuracy, and sensitivity parameters according to Center for Drug Evaluation and Research (CDER) guidance for Bio-analytical Method Validation (14).

The advantages of the present method include simple and single step extraction procedure using inexpensive chemicals and short run time. Also, this method was used to assay the carvedilol in plasma samples obtained from three patients with hypertension who had been given an oral tablet of Dilatrend (25 mg carvedilol).

Experimental

Chemicals and reagents

Carvedilol was obtained from Department of Cardiology, Faculty of Medicine, Ataturk University (Erzurum, Turkey). Atenolol as internal standard (IS) was kindly donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). *n*-Methyl-*n*-trimethylsilyl-trifluoroacetamide (MSTFA), ethylacetate, diethylether, and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO). Dilatrend tablet (25 mg carvedilol) was obtained Roche Pharmaceutical Industry (Istanbul, Turkey).

Apparatus and analytical conditions

Chromatographic analysis was carried out on an Agilent 6890N gas chromatography system equipped with 5973 series mass selective detector, 7673 series autosampler and Agilent chemstation. HP-5 MS column with 0.25 µm film thickness (30 m × 0.25 mm i.d., Santa Clara, CA) was used for separation.

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Splitless injection was used and the carrier gas was helium at a flow-rate of 1.5 mL/min. The injector volume was 1 µL. The injector and detector temperatures were 280°C. The oven temperature program was held at 230°C for 1 min, increased to 250°C at a rate of 25°C/min for 1 min and then increased to 315°C at a rate of 20°C/min for 5 min. The MS detector parameters were transfer line temperature 280°C, solvent delay 3 min, and electron energy 70 eV.

Preparation of stock and standard solutions

The stock solutions of carvedilol and IS were prepared in acetonitrile at concentration of 2500 ng/mL and stored at –20°C under refrigeration. Standard solutions of carvedilol (15, 50, 100, 200, 300, 400, and 500 ng/mL) were prepared by diluting with acetonitrile from stock solution. Also, quality control (QC) samples were prepared from stock solution at concentrations of 75, 250, and 450 ng/mL together with 500 ng/mL IS.

Sample preparation and derivatization procedure

MSTFA is an effective trimethylsilyl donor. *MSTFA* reacts to replace labile hydrogens on a wide range of polar compounds with a $–Si(CH_3)_3$ (TMS) group and is used to prepare volatile and thermally stable derivatives for GC–MS (15). In this study, the purpose of the derivatization reaction is the raise of sensitivity thus the possibility of working in low concentrations has been occurred. Therefore, carvedilol and IS were derivatized using MSTFA. The secondary amine (–NH) and hydroxy (–OH) groups, which render the compounds non-volatile and polar, were converted to the corrosponding silyl (–N-TMS) and (–O-TMS) groups, thereby rendering them volatile and non-polar.

A 1.0 mL aliquot plasma sample was transferred into 10-mL glass tube together with 0.2 mL IS solution (500 ng/mL) and 0.5 mL 1 M sodium hydroxide solution. After vortex mixing for 5 s, 4 mL of diethylether and ethylacetate was added (3:1, v/v), the mixture was vortexed for 30 s and then centrifuged at 3000 × *g* for 3 min. The organic layer was transferred into another tube and evaporated to dryness at room temperature under nitrogen gas.

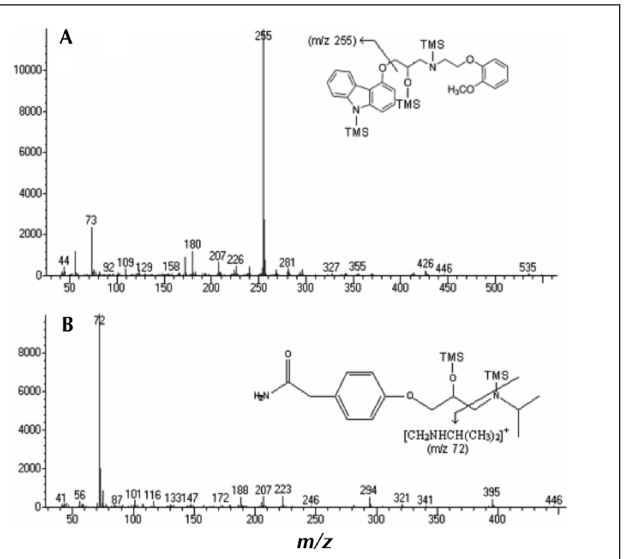

Figure 1. Chemical structures and full scan mass spectra of carvedilol (A) and atenolol (IS) (B) after derivatization with MSTFA.

The dry residue was dissolved in 100 µL of a mixture of acetonitrile and MSTFA (30:70, v/v). The mixture was vigorously shaken and then delayed at room temperature for 10 min. The mass spectra of the carvedilol and IS are shown in Figure 1. Then 1 µL of aliquot was injected into the GC–MS system.

Results

Method development

The method development for the assay of carvedilol was based on its chemical properties. Carvedilol is a polar molecule. Therefore, the capillary column coated with 5% phenyl, 95% dimethylpolysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations. Different temperature programs were investigated for GC oven. The end of this investigation, the best temperature program was selected for a good separation. The temperature programs of the GC oven was as follows: initial temperature 230°C for 1 min, increased to 250°C at a rate of 25°C/min for 1 min and then increased to 315°C at a rate of 20°C/min for 5 min. The splitless injection mode was chosen. To confirm the complete derivatization of carvedilol and IS, since only one peak appeared on the chromatogram, each compound was derivatized and analyzed separately. After establishing the optimum reaction conditions, the compounds were mixed together and then derivatized in order to perform a simultaneous analysis. Additionally, preliminary precision and linearity studies performed during the development of the method showed that the 1 µL injection volume was reproducible and the peak response was significant at the analytical concentration chosen.

Validation of the method

The validation of the method was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery, limit of detection (LOD), and limit of quantitation (LOQ) according to CDER guidance for Bio-analytical Method Validation (14).

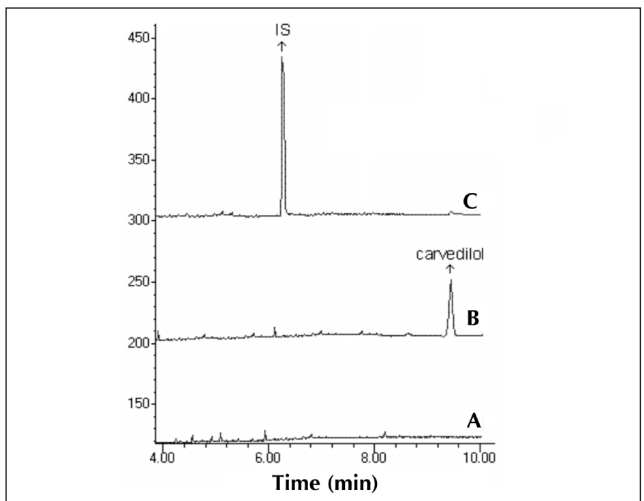

Figure 2. Typical SIM chromatogram of blank plasma (A), plasma spiked with 500 ng/mL carvedilol (B) and 500 ng/mL atenolol (IS) (C).

Specificity

The specificity of method was determined by checking the chromatograms obtained from blank plasma samples, and no endogenous interferences were encountered (Figure 2). The fragment ions (*m/z* 255 and 72) were used for quantitation of carvedilol and IS. The retention time of carvedilol-TMS and IS-TMS in human plasma was approximately 9.5 and 6.3 min.

Linearity

Seven different concentrations of carvedilol (15, 50, 100, 200, 300, 400, and 500 ng/mL) with constant concentration of IS (500 ng/mL) were spiked to the blank plasma as described previously. The calibration curves were established by plotting the ratio of the peak areas of carvedilol and IS obtained after extraction of the spiked plasma sample. The linear regression equation was calculated by the least squares method using Microsoft Excel program and summarized in Table I.

Precision and accuracy

Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability during the same day and intermediate precision on different days (3 days) were evaluated with six replicates of QC samples with 500 ng/mL IS. The accuracy of this analytic method was assessed as the percentage relative error (%RE). The accuracy and precision of the method were evaluated with QC samples at concentrations of 75, 250 and 450 ng/mL. The intra- and inter-day accuracy and precision results are shown in Table II. The intra- and inter-day precisions of the QC samples were satisfactory with RSD less than 8.0% and accuracy with RE within ± 11.0% (should be less than 15 according to CDER guidance for Bio-analytical Method Validation).

Table I. Linearity of Carvedilol in Human Plasma

Parameters	GC–MS
Linearity (ng/mL)	15–500
Regression equation*	y = 0.0033x + 0.0973
Standard deviation of slope	1.01×10^{-4}
Standard deviation of intercept	4.03×10^{-3}
Correlation coefficent	0.9955
Standard deviation of correlation coefficent	4.28×10^{-3}
Limit of detection (ng/mL)	5.0
Limit of quantitation (ng/mL)	15

* Based on 6 calibration curves. y = peak-area ratio; x = carvedilol conc. (ng/mL).

Table II. Precision and Accuracy of Carvedilol in Human Plasma

	Intra-day			Inter-day		
Added	Found ± SD*	Precision % RSD†	Accuracy§	Found ± SD*	Precision % RSD†	Accuracy§
75	78.6 ± 3.467	4.41	4.80	83.1 ± 4.745	5.71	10.80
250	241.2 ± 16.484	6.83	–3.52	255.6 ± 20.258	7.93	2.24
450	459.9 ± 9.229	2.01	2.20	458.5 ± 16.832	3.67	1.89

* SD = Standard deviation of six replicate determinations; RSD = Relative standard deviation.
† Average of six replicate determinations.
‡ Accuracy: (% relative error) (found – added) / added x 100.
§ Plasma volume (1.0 mL)

LOD and LOQ

The LOD is a parameter that provides the lowest concentration in a sample that can be detected from background noise but not quantitated. The limit of LOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy. The LOD and LOQ of the developed method were determined by injecting progressively low concentration of the standard solution under the chromatographic conditions. The LOD and LOQ were found to be 5.0 and 15 ng/mL, respectively (Table I).

Recovery

Recovery was performed to verify the effectiveness of the extraction step and the accuracy of the proposed method. The liquid–liquid extraction was used for the sample preparation in this work. Several solvents (ethylacetate, diethylether, dichloromethane, acetonitrile, butanol, and chloroform) were tested for the extraction. Finally, a diethylether and ethylacetate mixture (3:1, v/v) proved to be the most efficient in extracting carvedilol from human plasma. After extraction procedure, the dry residue was dissolved in 100 µL of a mixture of acetonitrile and MSTFA (30:70, v/v). The mixture was vigorously shaken and then delayed at room temperature for 10 min. Spiked plasma samples were prepared six times at all levels (15, 50, 100, 200, 300, 400, and 500 ng/mL) of the calibration graph of carvedilol. The recovery of carvedilol was determined by comparing the ratio of the amount of carvedilol and IS measured after analysis of spiked plasma samples with those found after direct injection of standard solutions at the same concentration levels. The analytical recovery of carvedilol from human plasma was given in Table III.

Matrix effect

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample (14). The matrix effect was investigated by comparing the amount of carvedilol and IS solutions with processed blank samples reconstituted with carvedilol and IS solutions. The blank plasmas used in this study were from five different batches of

Table III. Recovery of Carvedilol in Human Plasma

Added (ng/mL)	Found (Mean ± SD*)	% Recovery	% RSD†
15	12.93 ± 1.181	86.2	9.13
50	44.65 ± 3.456	89.3	7.74
100	90.10 ± 5.775	90.1	6.41
200	177.4 ± 10.18	88.7	5.74
300	268.8 ± 22.01	89.6	8.19
400	364.4 ± 31.81	91.1	8.73
500	429.5 ± 32.13	85.9	7.48

* Standard deviation of six replicate determinations; relative standard deviation.
† Average of six replicate determinations.

healthy human plasma. If the ratio < 85% or > 115%, a matrix effect was implied. The relative matrix effect of carvedilol at three different concentrations (150, 350, and 500 ng/mL) was less than ± 9.0% (Table IV). The results showed that there was no matrix effect of the analytes observed from the matrix of plasma in this study.

Stability

The stability of carvedilol in human plasma was assessed by analyzing low (200 ng/mL) and high (500 ng/mL) concentration level samples after storage for different times and temperatures. The short-term temperature stability was assessed by analyzing three aliquots of each of the low and high concentration samples at room temperature for 8 h. Freeze-thaw stability (–20°C in plasma) was checked through three cycles. Samples were stored at –20°C for 24 h and then thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h. Samples were analyzed after three freeze-thaw cycles. The long-term stability was assessed after storage at –20°C for 1 week. The results of the stability studies was given in Table V and no significant degradation of carvedilol was observed under the tested conditions.

Application of the method

Prior to the study, the clinical protocol was approved by the Ethics Committee of Faculty of Medicine, Ataturk University (2009/Number 41). All volunteer patients were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. The patients who submitted the agreements to attend this project were medically examined and three patients with hypertension were selected (38.2 ± 1.87 years; 76.1 ± 1.92 kg; 179 ± 5.21 cm) for pharmacokinetics study for carvedilol. The subjects were required to abstain from taking any other drug for 7 days prior the start of test. They were also forbidden to smoke or drink alcohol or beverages containing xanthine for 24 h before the beginning of the study until its end. Three patients with hypertension received an oral tablet (Dilatrend 25 mg) containing 25 mg of carvedilol. Then, they were allowed to drink water. The total amount of water drunk during the day was 1500 mL. The volunteers were sitting during lunch had normal activity (standing or sitting) during the study, but were never in a supine position during the 12 h after administration. Blood samples were taken into EDTA tubes at 0, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 h after oral administration. The blood was centrifuged 6000 × *g* for 10 min at ambient temperature, extracted, and derivatized with *MSTFA*. Representative chromatograms obtained before and after administration of the drug are shown in Figure 3.

Table IV. Matrix Effect Evaluation of Carvedilol and IS in Human Plasma (*n* = 3)*

Samples	Conc. level (ng/mL)	A (Mean ± SD)	B (Mean ± SD)	% Matrix effect
Carvedilol	150	138 ± 8.74	146 ± 6.22	94.5
	350	318 ± 15.75	327 ± 12.42	97.2
	500	432 ± 14.41	472 ± 11.34	91.5
IS	500	419 ± 13.57	447 ± 8.62	93.7

*(A) The amount of carvedilol and IS derivatized in blank plasma sample's reconstituted solution (the final solution of blank plasma after extraction and reconstitution), (B) The amount of carvedilol and IS derivatized with MSTFA.

Table V. Stability Data of Carvedilol in Human Plasma Under Various Storage Conditions (*n* = 3)

Conc. level (ng/mL)	Calculated conc. (ng/mL)	% RSD	% Relative error
Storage conditions: *Room temperature for 8 h*			
200	191	6.91	–4.50
500	475	7.98	–5.00
Storage conditions: *Three freeze– thaw cycles*			
200	187	7.83	–6.50
500	483	8.91	–3.40
Storage conditions: 1 week at –20 °C			
200	189	6.73	–5.50
500	481	9.14	–3.80

Discussion

Today, GC–MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples. Compared to HPLC, high-resolution capillary GC has been less frequently used. Because it reguires pre-conversion of multifunctional β-blockers into thermally stable volatile derivatives. However, it has inherently high resolving power and high sensitivity with excellent precision and accuracy allowed simultaneous detection of expected and unexpected β-blockers, their metabolites and contaminants (16).

During method development, it became evident that carvedilol and IS were very sensitive to matrix effects during the derivatization process in plasma. Sample preparation techniques, such as liquid–liquid extraction was used in order to minimise matrix suppression effects.

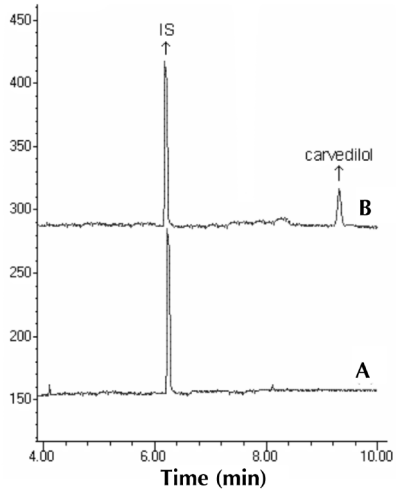

Figure 3. Typical SIM chromatogram of plasma obtained from a patient before (A) and after 1.5 h (B) oral administration of Dilatrend tablet (containing 25 mg carvedilol).

GC–MS method sensitivity is not enough for the determination of carvedilol in plasma. For this reason, *MSTFA* was chosen as a chromagenic derivatization reagent. In this study, the purpose of the derivatization reaction is the raise of sensitivity thus the possibility of working in low concentrations has been occurred.

Calibration curve of carvedilol was linear over the concentration range of 15–500 ng/mL for plasma which is as good as or superior to that reported in other paper (4).

Carvedilol was extracted from human plasma with a solid phase extraction procedure by Behn et al. (9). This method is also the most comprehensive method which can extract carvedilol in a single extraction procedure.

In this study, the recovery percentage of carvedilol is high (7,9,10,12); derivatization and extraction processes do not take much time (10,11); additionally, the retention time is short which is an advantage (7,8).

Borges et al. (12) have reported an LC–MS–MS for the analysis of carvedilol in human plasma. The calibration curve of LC–MS–MS method was linear for carvedilol in the range 0.1–200 ng/mL. Intra- and inter-day precision values were lower than 9.7%. The mean recovery of carvedilol was 80.8%. The LOQ of method was found 0.10 µg/mL. Detection using LC–MS–MS would be a more sensitive approach but is costly and not yet available for every laboratory.

Also, in statistical comparison ($p > 0.05$) with other methods in the literature (4,8,9) the proposed method has indicated high accuracy and recovery.

Conclusion

In the present work, a simple and sensitive GC–MS method has been developed for the determination of carvedilol in human plasma. The method was completely validated by using stability, specificity, linearity, sensitivity, accuracy, and precision parameters for determination of carvedilol in human plasma. Also, the extraction and derivatization procedures in this study were simple. No significant interferences and matrix effect caused by endogenous compounds were observed.

To the authors' knowledge, this is the first description of carvedilol analysis in human plasma by GC–MS method in the literature. It can be very useful and an alternate to performing pharmacokinetic studies in determination of carvedilol for clinical use.

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