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Gas chromatograph–mass spectrometric method for the determination of carvedilol and its metabolites in human urine

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Abstract

A sensitive and efficient method was developed for the determination of carvedilol and its metabolites in human urine by gas chromatography–mass spectrometry (GC–MS). Urine samples were hydrolyzed with β-glucuronidase/arylsulfatase (from *Helix* pomatia) and the target compounds were extracted with liquid–liquid extraction. The extracts were completely derivatized with MSTFA and MBTFA and analyzed by GC–MS using an Ultra-2 column. The linearity of the assay ranges were 0.75–75 ng mL^{-1} for carvedilol and *o*-desmethyl carvedilol (*o*-DMC), and 3.0–75 ng mL^{-1} for 4-hydroxyphenyl carvedilol (4-HPC) and 5-hydroxyphenyl carvedilol (5-HPC). The absolute recovery of carvedilol and its metabolites added to a blank urine sample was 80.1–97.8%. The limits of detection (LOD) and quantitation (LOQ) of carvedilol and *o*-DMC were 0.30 and 0.75 ng mL^{-1}, and its of 4-HPC and 5-HPC were 0.75 and 3.0 ng mL^{-1}, respectively. The reproducibilities were 1.86–11.5% for the intra-day assay, and 0.70–1.71% for the inter-day assay precision and the degree of inaccuracy was −3.0 to 3.9% at the concentration of 75 ng mL^{-1}. The proposed GC–MS method was effective for the determination of carvedilol and its three metabolites in human urine.

Keywords: Carvedilol; GC–MS; Metabolites

1. Introduction

Carvedilol, (±)-1-(carbazol-4-yloxy)-3-((2-(*o*-methoxyphenoxy)ethyl)amino)-2-propanol, is a nonselective β-blocking agent [1–2], which also has vasodilating properties that are mainly attributed to its blocking activity at α_1-receptors [3]. Carvedilol is used in the treatment of mild to moderate hypertension [4–5] and angina pectoris [6], and is often used in combination with other drugs. Carvedilol is also sometimes used by athletes for performance enhancement. However, the administration of this drug can injure an athlete's health, and obviously acts against the spirit of equality of opportunity and, thus, against the ethics of the sport. Carvedilol is classified as a prohibited drug by the World Anti-Doping Agency (WADA) [7] and the International Olympic Committee (IOC). Carvedilol is readily converted in vivo to the corresponding metabolites, which are *o*-desmethyl carvedilol (*o*-DMC), 4-hydroxyphenyl carvedilol (4-HPC) and 5-hydroxyphenyl carvedilol (5-HPC) (Fig. 1) [8–10]. The currently available methods for the determination of carvedilol in plasma, urine and other biological materials are focused on the separation of the enantiomers [11], liquid–liquid or liquid–solid extraction using high performance liquid chromatography (HPLC) [12–13] or capillary electrophoresis (CE) [14–16]. There is no literatures, which were performed the determination of carvedilol and its metabolites using GC–MS. Therefore, in this study, we present a new method of analyzing carvedilol and its three metabolites simultaneously using gas chromatography (GC)–mass spectrometry (MS).

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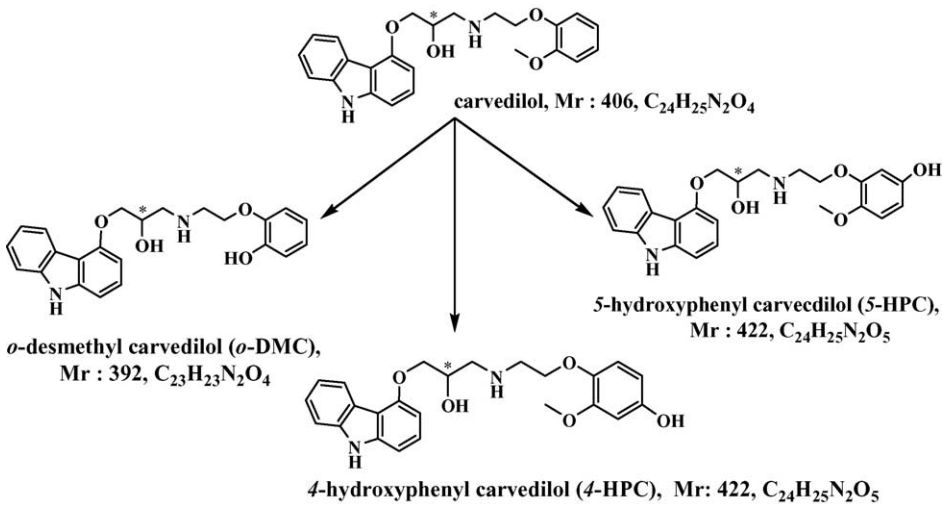

Fig. 1. Chemical structures of carvedilol and its three metabolites.

2. Experimental procedure

2.1. Chemicals

Carvedilol, *o*-DMC, 4-HPC and 5-HPC (purity: 99.9%) were obtained from Toronto Research Chemicals, Inc. (Ont., Canada). Carazolol (internal standard) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol and acetonitrile were obtained in HPLC grade from J.T. Baker Chemical Co. (NJ, USA) and all other reagents were of analytical or HPLC grade. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and *N*-methyl-*bis*-trifluoracetamide (MBTFA) were obtained from Pierce (Rockford, IL, USA). The stock solutions of carvedilol and its metabolites were made by dissolving 1 mg of the substance in 10 ml of methanol. β-Glucuronidase/arylsulfatase (from *Helix* pomatia) was obtained from Roche Diagnostics Co. (Mannheim, Germany).

2.2. Gas chromatographic–mass spectrometric (GC–MS) analysis

After being derivatized and extracted from human urine, carvedilol and its metabolites were analyzed using an Agilent 6890N Gas Chromatograph combined with an Agilent 5973N Mass Selective Detector (GC–MSD, Palo Alto, CA, USA). The ionization mode was electron impact (EI) with a 70 eV beam of electrons and the mass range of the scan mode was *m*/*z* 40–750. The capillary columns used were Ultra-1 (100% dimethylpolysiloxane) and Ultra-2((5%-phenyl)-methylsiloxane) (Agilent, Palo Alto, CA, USA) with a length of 17 m, an inner diameter of 0.20 mm, a film thickness of 0.33 μm, a carrier gas flow rate of 1.0 mL min^{-1} (He), and the injection was made on the split mode (1:10). The temperature programming of the column was as follows: 200–300 °C at 20 °C min^{-1} and holding for 20 min. The temperature of the injector and transferline were 280 and 300 °C, respectively.

2.3. Sample preparation and derivatization

Five milliliters of the urine sample was transferred to a centrifuge tube and 4 μL of 20 μg mL^{-1} carazolol (ISTD) and 1 mL of 6 M hydrochloric acid or 50 μL of β-glucuronidase/arylsulfatase (from *Helix* pomatia) with 4.5 U/mL and 14 U/mL at 37 °C were added for the purpose of hydrolysis. The tube was shaken to homogenize the mixture and heated at 105 °C for 30 min (acid hydrolysis) or 55 °C for 3 h (enzyme hydrolysis). After cooling, the pH of the sample (pH 7.0) was adjusted with a buffer system (Na_2CO_3:$NaHCO_3$ = 1:2), KOH and HCl solution, and then 0.5 mL of *t*-butanol, 3 g of anhydrous sulfate and 5 mL of diethyl ether were added. The tube was shaken mechanically for 20 min at 175 min^{-1} and centrifuged at 2096 × *g* for 5 min. The ethereal phase was transferred to another tube and evaporated to dryness under a stream of nitrogen. The residue obtained was dissolved in 50 μL of acetonitrile, and 90 μL of MSTFA was added. The tube was heated at 80 °C for 5 min and then cooled to room temperature. 10 μL of MBTFA was added and the tube was heated at 80 °C for 10 min. Two microliters of the resulting solution was injected into the GC–MS system.

2.4. Calibration curves

The calibration curves were obtained by spiking the control urine with 100 μg mL^{-1} of the stock solution. The concentrations of the spiked analytes in urine were 0.75, 3.0, 12, 24, 48 and 75 ng mL^{-1}. The calibration curves were made from the peak area ratio of carvedilol or its metabolites to the internal standard. The *m*/*z* 255 was used for quantitation of

carvedilol and its metabolites and the peak area of the SIM mode was used.

2.5. Recovery test

Four microliters of ISTD (20 μg mL^{-1}), and carvedilol and its three metabolites at concentrations of 0.10 and 2.5 μg mL^{-1}, were placed into a test tube. The solvent was removed with a nitrogen stream and 50 μL of acetonitrile was added. Ninety microliters of MSTFA was added and the tube was heated at 80 °C for 5 min and cooled at room temperature. Ten microliters of MBTFA was added and the tube was heated at 80 °C for 10 min. The absolute recoveries of carvedilol and its three metabolites were calculated by comparing the amounts of the compounds present in the final solution after covering the whole method (described in Section 2.3) with the amounts that were injected after the sample preparation procedure described above.

2.6. Drug administration and sample collection

After the oral administration of a capsule containing 25 mg of carvedilol (Dilatrend^{TM}, Chong Kun Dang Pharmaceutical Co., Ltd.), urine samples were collected at the following times (volume of urine): 0 (625 mL), 3 (500 mL), 6 (125 mL), 12 (300 mL), 18 (375 mL), 26 (400 mL), 38 (500 mL), 41 (500 mL) and 50 (450 mL) h. The volunteer was a man who is 27 years old and 70 kg weight. The samples were stored in a refrigerator at 4 °C.

3. Results and discussion

3.1. Derivatization and separation

To increase the performance of the gas chromatographic separation, the extracts were derivatized using MSTFA and MBTFA. The secondary amine (–NH) and hydroxy (–OH) groups, which render the compounds non-volatile and polar, were converted to the corresponding silyl (–N–TMS and –O–TMS) or acyl (–N–TFA) groups, thereby rendering them volatile and non-polar. The amine group located in the carbazol moiety was derivatized into the trimethylsilyl (–TMS) group, while the one, which is located in the amino propanol moiety was derivatized into the trifluoroacetyl (–TFA) group. To confirm the complete derivatization of each compound, since only one peak appears 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. The GC–MS chromatograms of the derivatized carvedilol and its metabolites obtained using the Ultra-1 and Ultra-2 columns are shown Fig. 2. Carvedilol and *o*-DMC were detected at the retention times of 13.74 and 13.46 min, respectively. Since these two peaks were eluted with similar retention times, they could not be completely separated on the Ultra-1 column. However, the Ultra-2 column, which consists of a more polar stationary phase, provided for the complete separation for the derivatized carvedilol and the three metabolites, and the elution order of carvedilol and *o*-DMC was changed on this column,

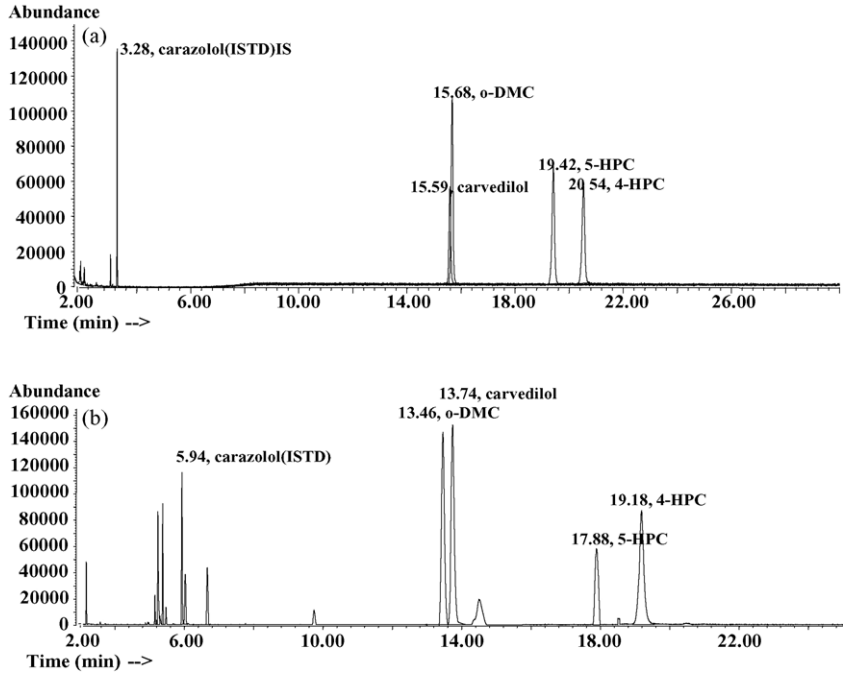

Fig. 2. GC–MS chromatograms of the derivatized carvedilol and its three metabolites: (a) Ultra-1 column and (b) Ultra-2 column.

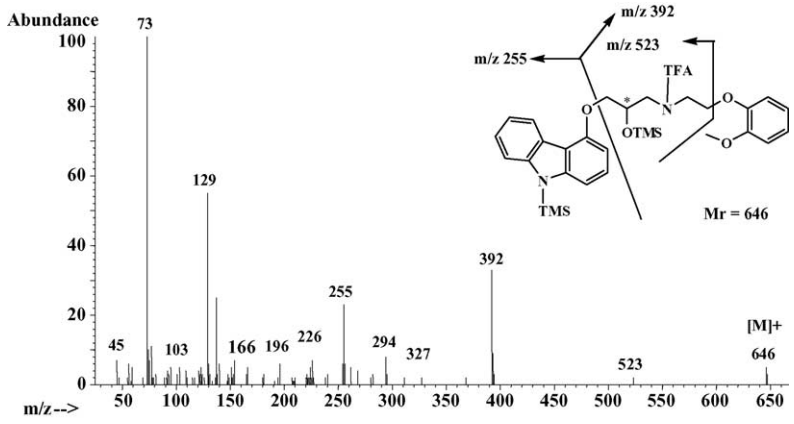

Fig. 3. Mass spectrum of the derivatized carvedilol.

with carvedilol, *o*-DMC, 4-HPC and 5-HPC being detected at the retention times of 13.74, 13.46, 19.18 and 17.88 min, respectively.

3.2. Mass spectra

The mass spectra of the derivatized carvedilol and its three metabolites demonstrated the presence of the various common characteristic ions and each molecular ion. The mass spectrum of the derivatized carvedilol is shown in Fig. 3. The molecular ion was detected at *m*/*z* 646 and the characteristic ions at *m*/*z* 255 (carbazolyl-amino-TMS), 392 and 523 (cleavage of methoxyphenoxy group). The mass spectrum of the derivatized *o*-DMC is shown in Fig. 4. The molecular ion was detected at *m*/*z* 704 and the characteristic ions are *m*/*z* 255 (carbazolyl-amino-TMS), 450 and 523 (cleavage of phenoxy-oxy-TMS group). The mass spectrum of the derivatized 4-HPC and are shown in Figs. 5 and 6, respectively. The molecular ion was detected at *m*/*z* 734 and the characteristic ions are *m*/*z* 255 (carbazolyl-amino-TMS), 480 and 523 (cleavage of methoxy-para-oxy-TMS-phenoxy group for 4-HPC and methoxy-meta-oxy-TMS-phenoxy group for 5-HPC). The gas chromatographic retention times and characteristic ions of carvedilol, *o*-DMC, 4-HPC and 5-HPC, are shown in Table 1.

Table 1
GC–MS retention times and characteristic ions of carvedilol and its three metabolites using Ultra-2 column

Compounds	Retention time (min)	Characteristic ions (*m*/*z*)
Carvedilol	13.74	129, 255, 294, 392, 523, 646 (M^+)
o-DMC	13.46	129, 255, 294, 450, 523, 704 (M^+)
5-HPC	19.18	129, 255, 294, 480, 523, 734 (M^+)
4-HPC	17.88	129, 255, 294, 480, 523, 734 (M^+)

3.3. Hydrolysis

To obtain the free form of the phase II metabolites present in the human urine samples, enzymatic hydrolysis and acidic hydrolysis were carried out. An acidic hydrolysis process with 6 M HCl at 105 °C for 30 min resulted in the degradation of carvedilol. This was confirmed by the observation that the chromatogram obtained from the acidic hydrolysis showed a lower peak area than that obtained from the enzymatic hydrolysis with β-glucuronidase/arylsulfatase(from *Helix* pomatia) at 55 °C for 3 h (Fig. 7).

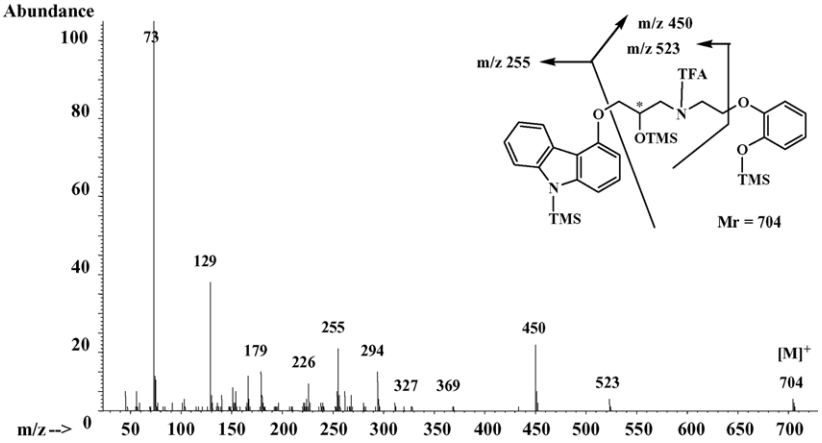

Fig. 4. Mass spectrum of the derivatized *o*-DMC.

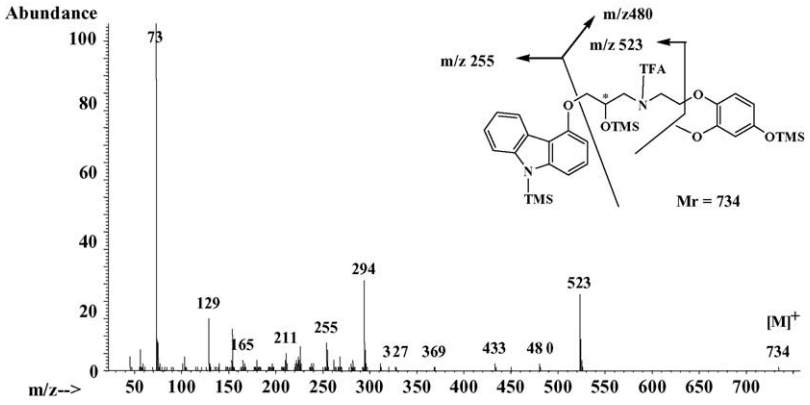

Fig. 5. Mass spectrum of the derivatized 4-HPC.

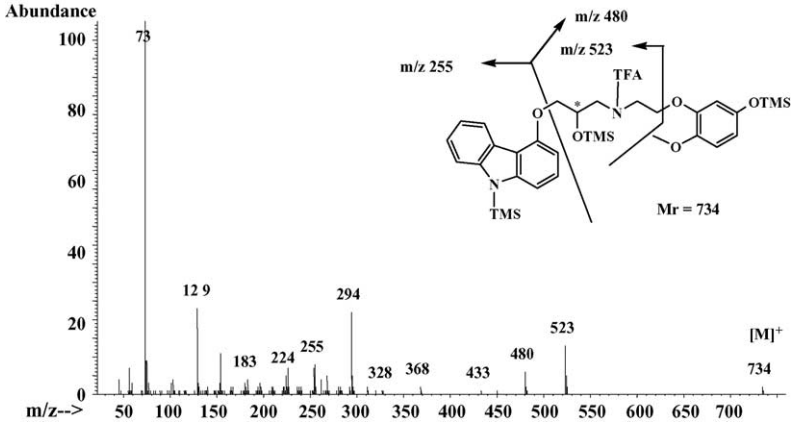

Fig. 6. Mass spectrum of the derivatized 5-HPC.

3.4. Validation

SIM mode for the data collection was used. The calibration curves were linear in the range of 0.75–75 ng mL^{-1} for carvedilol and *o*-DMC, and 3.0–75 ng mL^{-1} for 4-HPC and 5-HPC. The equations corresponding to the calibration curves, consisting of five points for carvedilol, *o*-DMC, 4-HPC and 5-HPC, $y = 0.5199x + 1.6235$ ($r^2 = 0.9917$), $y = 0.5668x - 0.3464$ ($r^2 = 0.9950$), $y = 0.3292x - 1.7629$ ($r^2 = 0.9930$), $y = 0.2269x - 1.0050$ ($r^2 = 0.9958$), respectively (Fig. 8). The limits of detection (LOD) was defined as the lowest concentration at which the signal to noise ratio were above 3, and limit of quanttation (LOQ) was defined as a concentration where the accuracy and precision lies within 20% limits. The LOD and LOQ in the urine sample were 0.30 and 0.75 ng mL^{-1} for carvedilol, 0.30 and 0.75 ng mL^{-1}

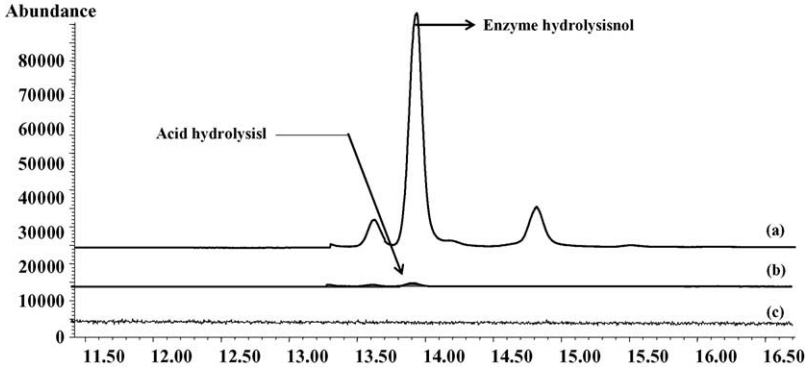

Fig. 7. Comparison between enzymatic and acidic hydrolysis for the treatment of the spiked urine samples of carvedilol using Ultra-2 column: (a) after enzyme hydrolysis; (b) after acid hydrolysis and (c) no hydrolysis.

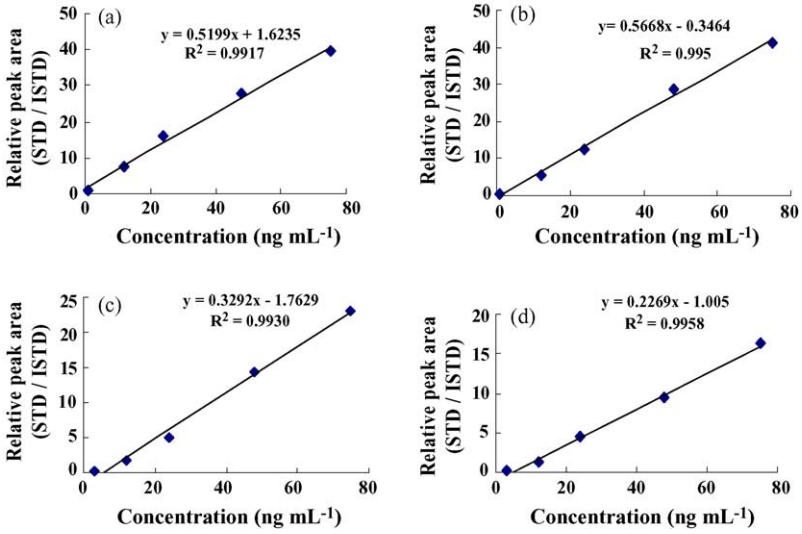

Fig. 8. Calibration curves of carvedilol and its three metabolites.

Table 2
LOD and LOQ of carvedilol and its three metabolites ($n = 4$)

Compounds	LOD		LOQ	
	Mean (ng mL^{-1})	Percentage R.S.D.	Mean (ng mL^{-1})	Percentage R.S.D.
Carvedilol	0.30	14.0	0.75	8.77
o-DMC	0.30	19.1	0.75	4.14
4-HPC	0.75	12.0	3.0	15.4
5-HPC	0.75	5.83	3.0	12.0

for *o*-DMC, 0.75 and 3.0 ng mL^{-1} for 4-HPC, and 0.75 and 3.0 ng mL^{-1} for 5-HPC in urine sample, respectively (Fig. 9 and Table 2).

The intra-assay and inter-assay precision of the proposed method is shown in Tables 3 and 4. Three sets of quality control samples (3.0, 24 and 75 ng mL^{-1}) were analyzed along with calibration samples in one batch. The precisions for the intra-day and inter-day assay were 0.60–63.4 and 0.463–4.78% (R.S.D.), respectively. The degree of inaccuracies for the intra-day and inter-day assay were −101 to 72.5 and −100 to 57.2% (bias) at a concentration of 3.0, 24 and 75 ng mL^{-1}. The accuracies of the 4-HPC and 5-HPC were not good at the low concentration (below 24 ng mL^{-1}).

The absolute recoveries to a blank urine sample at concentration of 75 ng mL^{-1} were 97.8% for carvedilol, 87.4% for *o*-DMC, 94.0% for 4-HPC, and 80.1% for 5-HPC (Table 4).

3.5. Concentration-time profile

The GC–MS chromatograms of the derivatized carvedilol and its metabolites obtained from human urine are shown in Fig. 10. The assayed peaks were completely separated. The concentration versus time profile of carvedilol and its three metabolites, following the administration of a dose of 25 mg of carvedilol in human urine, are shown in Fig. 11. 24% of the dose of carvedilol was excreted in the urine, in the form of carvedilol and its three metabolites, within 41 h (Table 5).

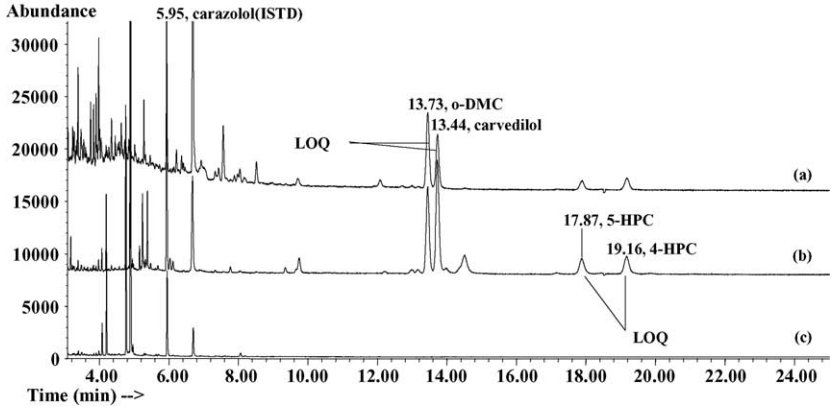

Fig. 9. Chromatograms (Ultra-2 column) for the limit of quantitation (LOQ) for carvedilol and *o*-DMC (0.75 ng mL^{-1}), 4-HPC and 5-HPC (3.0 ng mL^{-1}) and blank urine.

Table 3
Intra- and inter-assay precision and accuracy of carvedilol and *o*-DMC ($n = 4$)

	Nominal concentration of carvedilol and *o*-DMC in urine, calibration range 3.00–75.0 (ng mL^{-1})					
	3.00		24.0		75.0	
	Carvedilol	*o*-DMC	Carvedilol	*o*-DMC	Carvedilol	*o*-DMC
Concentration found (mean value) (ng mL^{-1})						
Day 1 ($n = 4$)	2.09	0.82	22.6	27.7	73.5	72.7
Day 2 ($n = 4$)	2.27	1.07	22.5	27.4	72.3	72.1
Day 3 ($n = 4$)	2.16	1.96	22.4	27.2	72.9	72.4
Inter-assay ($n = 3$)	2.17	1.28	22.5	27.4	72.9	72.4
Bias (mean value) (%)						
Day 1 ($n = 4$)	30.2	72.5	5.75	−15.5	1.98	3.03
Day 2 ($n = 4$)	24.3	64.3	6.33	−14.1	3.56	3.92
Day 3 ($n = 4$)	28.1	34.8	6.87	−13.3	2.77	3.48
Inter-assay ($n = 3$)	27.6	57.2	6.32	−14.3	2.77	3.48
Precision (mean value) (%R.S.D.)						
Day 1 ($n = 4$)	12.1	12.4	19.2	19.4	7.21	6.58
Day 2 ($n = 4$)	40.2	6.90	14.9	13.9	2.79	2.45
Day 3 ($n = 4$)	8.53	10.7	0.742	3.02	2.10	2.12
Inter-assay ($n = 3$)	4.08	46.3	0.601	0.962	0.813	0.463

Table 4
Intra- and inter-assay precision and accuracy of 4-HPC and 5-HPC ($n = 4$)

	Nominal concentration of 4-HPC and 5-HPC in urine, calibration range 3.00–75.0 (ng mL^{-1})					
	3.00		24.0		75.0	
	4-HPC	5-HPC	4-HPC	5-HPC	4-HPC	5-HPC
Concentration found (mean value) (ng mL^{-1})						
Day 1 ($n = 4$)	6.02	5.36	20.7	24.2	75.5	76.2
Day 2 ($n = 4$)	5.96	5.88	20.9	22.6	74.8	77.3
Day 3 ($n = 4$)	6.02	5.53	20.8	23.2	73.0	76.7
Inter-assay ($n = 3$)	6.00	5.59	20.8	23.3	74.4	76.7
Bias (mean value) (%)						
Day 1 ($n = 4$)	−101	−78.6	14.0	−0.763	−0.648	−1.61
Day 2 ($n = 4$)	−98.8	−96.1	12.9	5.89	0.317	−3.05
Day 3 ($n = 4$)	−101	−84.4	13.4	3.13	2.66	−2.33
Inter-assay ($n = 3$)	−100	−86.4	13.4	2.75	0.776	−2.33
Precision (mean value) (%R.S.D.)						
Day 1 ($n = 4$)	22.2	10.4	12.3	15.9	7.04	11.5
Day 2 ($n = 4$)	13.5	16.9	63.4	14.1	3.28	2.87
Day 3 ($n = 4$)	7.11	14.0	3.20	4.81	1.86	3.30
Inter-assay ($n = 3$)	0.584	4.78	0.619	3.44	1.71	0.704

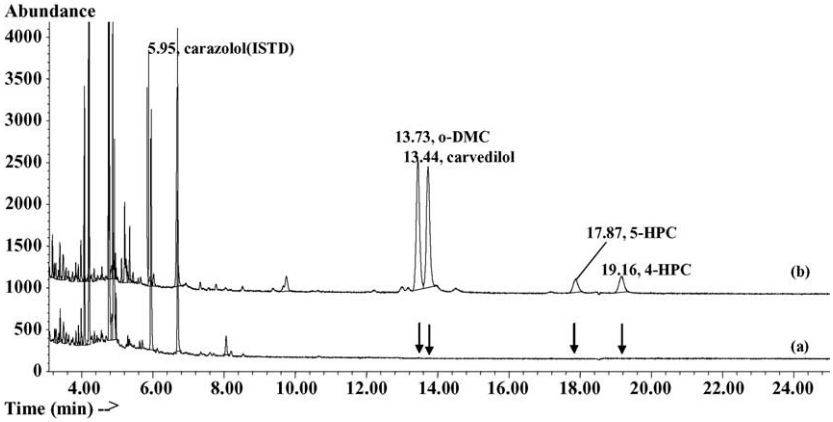

Fig. 10. GC–MS chromatogram (Ultra-2 column) of the derivatized carvedilol and its three metabolites in human urine: (a) blank urine and (b) dosed urine.

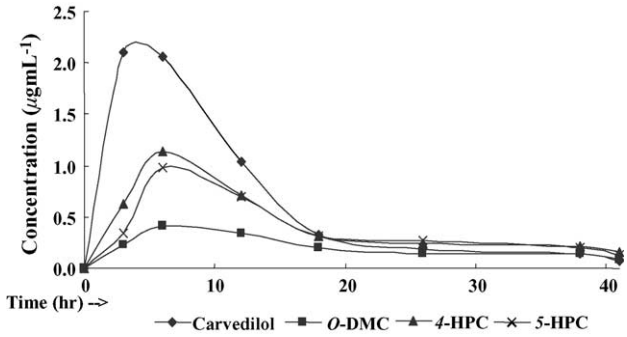

Fig. 11. Assay of carvedilol and its three metabolites in human urine after a single oral administration of carvedilol at a dose of 25 mg.

Table 5
The absolute recovery of carvedilol and its three metabolites ($n = 4$)

Compounds	Concentration (ng mL^{-1})	Mean ± S.D. (%R.S.D.)
Carvedilol	3	107 ± 0.045 (12.1)
	75	119 ± 0.136 (7.21)
o-DMC	3	83.3 ± 0.269 (12.4)
	75	75.1 ± 0.217 (6.58)
4-HPC	3	80.1 ± 5.28 (22.2)
	75	94.0 ± 5.12 (7.04)
5-HPC	3	87.4 ± 9.53 (10.4)
	75	97.8 ± 11.8 (11.5)

4. Conclusion

An analytical method was developed for the measurement of carvedilol and its metabolites in human urine by gas chromatography–mass spectrometry (GC–MS). The compounds were completely separated on the GC–MS chromatogram when using an Ultra-2 column. This method gave good precision and accuracy for the determination of carvedilol and its three metabolites and can be used for the drug testing of sports athletes.

Acknowledgement

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