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# Development and Validation of GC–MS Method for Determination of Metoprolol in Human Urine

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## Abstract

**This paper describes a specific, sensitive, and accurate gas chromatography–mass spectrometry method for determination of metoprolol in human urine after derivatization with *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). The method employed a one-step extraction of metoprolol from human urine with a mixture of ethylacetate and diethylether (2:1, v/v) at basic pH using atenolol as internal standard. Calibration curves were linear over the concentration range 50–3000 ng/mL. Intra- and inter-day precision values for metoprolol in human urine were less than 6.0%. The analytical recovery of metoprolol from human urine averaged 90.88%. The limits of detection and quantification of metoprolol were 5.0 and 15 ng/mL, respectively. Also, the developed and validated method was successfully applied to a patient with hypertension who had been given an oral tablet of 100 mg metoprolol.**

## Introduction

Metoprolol, 1-(isopropylamino)-3-[p-(2-methoxyethyl)phenoxy]-2-propanol, is a kind of β adrenaline receptor blocker. It is widely used for the treatment of hypertension, angina, miocardial infarction, arrhythmia, hyperthyroidism, and other related diseases (1,2).

Several methods have been reported for determination of metoprolol including gas chromatography–mass spectrometry (GC–MS) (3–5), high-performance liquid chromatography (HPLC) (2,6–10), LC–MS (11–13), and LC–MS–MS (14) in human urine and other biological fluids.

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

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In addition, no method is reported to date for determination of metoprolol by GC–MS from a patient with hypertension who had been given metoprolol. Therefore, we report a GC–MS method for the determination of metoprolol after a derivatization procedure in human urine using internal standard methodology.

The developed method was validated by using linearity, stability, precision, accuracy, and limits of detection (LOD) and quantification (LOQ) parameters, according to International Conference on Harmonization (ICH) guidelines (15).

The advantages of present method include simple and single-step extraction procedure using inexpensive chemicals and short run-time. Also, this method was used to assay the metoprolol in urine samples obtained from a patient with hypertension who had been given an oral tablet of Problok (100 mg metoprolol).

## Experimental

### Chemicals and reagents

Metoprolol tartrate, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), ethylacetate, dichloromethane, acetonitrile, diethylether, and chloroform were purchased from Sigma Aldrich (St. Louis, MO). Atenolol as internal standard (IS) was kindly donated from Abdi Ibrahim Pharmaceutical Industry (Istanbul, Turkey). Problok tablet (100 mg metoprolol tartrate) was obtained Terra Pharmaceutical Industry (Istanbul, Turkey).

### Apparatus and analytical conditions

Chromatographic analysis was carried out on an Agilent 6890N GC system equipped with 5973 series mass selective detector, 7673 series autosampler, and Agilent Chemstation (Palo Alto, CA). An Agilent HP-5 MS column with 0.25-µm film thickness (30 m × 0.25 mm i.d.) was used for separation. Splitless injection was used, and the carrier gas was helium at a flow rate of 1 mL/min. The injector and detector temperatures were 280°C. The MS detector parameters were transfer line temperature 280°C, solvent delay 3 min, and electron energy 70 eV. The oven temperature program was held at 150°C for 1 min, increased to 220°C at a rate of 20°C/min for 1 min, and then increased to 300°C at a rate of 10°C/min for 1 min.

### Preparation of stock and standard solutions

The stock solutions of metoprolol and IS were prepared in acetonitrile at concentration of 5000 and 2500 ng/mL, respectively. Standard solutions of metoprolol (50, 100, 200, 300, 400, 500, 1000, 2000, and 3000 ng/mL) were prepared by diluting with acetonitrile from stock solution. Also, quality control (QC) samples were prepared from stock solution at concentrations of 150, 750, and 2500 ng/mL.

**Table I. Linearity of Metoprolol in Human Urine**

| Parameters | GC–MS |
|---|---|
| Linearity (ng/mL) | 50–3000 |
| Regression equation* | $y = 0.0029x + 0.2791$ |
| Standard deviation of slope | $5.77 \times 10^{-4}$ |
| Standard deviation of intercept | $6.70 \times 10^{-3}$ |
| Correlation coefficent | 0.9938 |
| Standard deviation of correlation coefficent | 2.64 x 10-4 |
| Limit of detection (ng/mL) | 5.0 |
| Limit of quantification (ng/mL) | 15 |

* Based on six calibration curves, where $y$ = peak-height ratio and $x$ = metoprolol concentration (ng/mL).

**Table II. Precision and Accuracy of Metoprolol in Human Urine**

| | Intra-day | | | Inter-day | | |
|---|---|---|---|---|---|---|
| Added | Found ± SD* | Precision (%RSD)† | Accuracy‡ | Found ± SD* | Precision (%RSD)† | Accuracy‡ |
| *Urine Pools* | | | | | | |
| 150 | 154.2 ± 6.396 | 4.148 | 2.800 | 154.5 ± 8.582 | 5.555 | 3.000 |
| 750 | 754.2 ± 7.874 | 1.044 | 0.5600 | 767.5 ± 17.97 | 2.341 | 2.333 |
| 2500 | 2480 ± 52.81 | 2.129 | –0.800 | 2516 ± 83.59 | 3.322 | 0.640 |

* Standard deviation of six replicate determinations.
† Average of six replicate determinations.
‡ Accuracy = (% relative error) (found – added)/added × 100.
§ Urine volume = 0.5 mL.

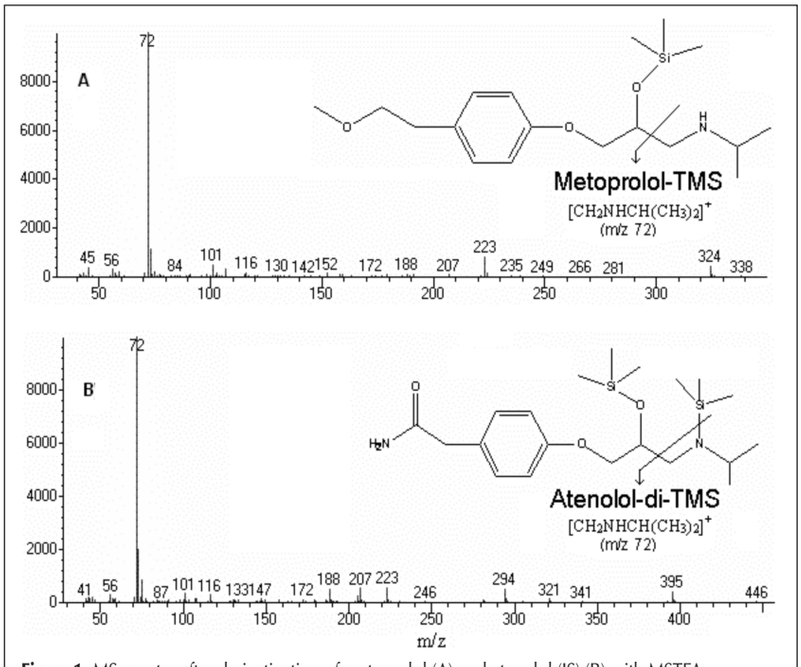


**Figure 1.** MS spectra after derivatization of metoprolol (A) and atenolol (IS) (B) with MSTFA.

### Sample preparation and derivatization procedure

MSTFA is an effective trimethylsilyl (TMS) donor. MSTFA reacts to replace labile hydrogens on a wide range of polar compounds with a TMS group and is used to prepare volatile and thermally stable derivatives for GC–MS (16). To increase the performance of the GC separation, metoprolol and IS were derivatized using MSTFA (Figure 1). The secondary amine (-NH) and hydroxy (-OH) groups were converted to the corrosponding silyl (-N-TMS) and (-O-TMS) groups. =

A 0.5 mL of the urine sample was transferred to a 10-mL centrifuge tube. One-tenth milliliter standard metoprolol solutions together with 0.2 mL IS solution (500 ng/mL) and 0.5 mL 1 M sodium hydroxide solution were added. The solutions were briefly vortexed. Then, 3 mL ethylacetate and diethylether mixture (2:1, v/v) was added, vortexed for 30 s, and centrifuged at $3000 \times g$ for 7 min. The supernatant was transferred into another centrifuge tube and evaporated to dryness at room temperature under nitrogen gas.

The dry residue was dissolved in 100 µL of a mixture of acetonitrile and MSTFA (50:50, v/v). The mixture was vigorously shaken and then delayed at room temperature for 10 min. One microliter sample was injected into the GC–MS system.

## Results

### Validation of the method

The validation was carried out by establishing specifity, linearity, intra- and inter-day precision, accuracy, recovery, LOD, and LOQ, according to ICH (15) and Center for Drug Evaluation and Research (CDER) guidance for Bioanalytical Method Validation (17).

### Specificity

The specificity of method was studied by checking the chromatograms obtained from blank urine samples. Each blank sample should be tested for interference, and no endogenous interference was encountered (Figure 2A). The fragment ion $[CH_2NHCH(CH_3)_2]^+$ (*m/z* 72) was used for quantification of metoprolol and IS. The retention time of metoprolol-TMS and IS-di-TMS in human urine was approximately 7.8 and 10.6 min, respectively (Figure 2B).

### Linearity

Nine different concentrations of metoprolol (50, 100, 200, 300, 400, 500, 1000, 2000, and 3000 ng/mL) with constant concentration of IS (500 ng/mL) were spiked to the blank urine as described previously. The calibration curves were established by plotting the ratio of the peak height of metoprolol and IS obtained after extrac-

tion and derivatization. The calibration curves were found to be linear over an analytical range of 50–3000 ng/mL. The linear regression equation was calculated by the least squares method using Microsoft Excel program and is summarized in Table I.

### Precision and accuracy

Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability on the same day and intermediate precision on different days (three days) were evaluated with six replicates of QC samples with 500 ng/mL IS. The intra-day relative standard deviation (RSD) was < 4.15%, and the inter-day RSD was < 5.56% for human urine. The accuracy of this method was assessed as the percentage relative error and relative error for accuracy was ≤ 3.00% (Table II).

### LOD and LOQ

The LOD is the lowest amount of metoprolol in a sample that can be detected but not necessarily quantitated as an exact value. The LOQ is the lowest amount of metoprolol that can be quantitatively determined with suitable precision. The LOD and LOQ of the developed method were determined by injecting progressively low concentration of the standard solution under the chromatographic conditions. The lowest concentrations assayed where the signal-to-noise ratio was at least 10:1; this concentration was regarded as LOQ. The LOD was defined as a signal-to-noise ratio of 3:1. The LOD and LOQ values for metoprolol 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, ethylacetate and diethylether mixture (2:1, v/v) proved to be the most efficient in extracting metoprolol from human urine. After extraction procedure, the dry residue was dissolved in 100 µL of a mixture of acetonitrile and MSTFA (50:50, v/v). The mixture was vigorously shaken and then delayed at room temperature for 10 min. Spiked urine samples were prepared six times at all levels (50, 100, 200, 300, 400, 500, 1000, 2000, and 3000 ng/mL) of the calibration graph of metoprolol. The recovery of metoprolol was determined by comparing the ratio of the amount of metoprolol and IS measured after analysis of spiked urine samples with those found after direct injection of standard solutions at the same concentration levels. The analytical recovery of metoprolol from human urine is given in Table III.

### Matrix effect

The matrix effect is defined as the direct or indirect alteration or interference in response to the presence of unintended analytes or other interfering substances in the sample (17). The matrix effect was investigated by comparing the amount of metoprolol and IS solutions with processed blank samples reconstituted with metoprolol and IS solutions. The blank urines used in this study were from five different batches of healthy human urine. If the ratio was < 85% or > 115%, a matrix effect was implied. The relative matrix effect of metoprolol at three different concentrations (150, 750, and 3000 ng/mL) was less than ± 11% (Table IV). The results showed that there was no matrix effect of the analytes observed from the matrix of urine in this study.

### Stability

The stability of metoprolol in human urine was assessed by analyzing low (500 ng/mL) and high (2500 ng/mL) concentration level samples after storage for different times and temperatures. The short-term temperature stability was assessed by

**Table III. Recovery of Metoprolol in Human Urine**

| Added (ng/mL) | Found (mean ± SD)* | % Recovery | % RSD† |
|---|---|---|---|
| 50 | 45.36 ± 2.082 | 90.72 | 4.589 |
| 100 | 92.43 ± 4.965 | 92.43 | 5.372 |
| 200 | 180.3 ± 10.39 | 90.15 | 5.746 |
| 300 | 268.9 ± 10.22 | 89.63 | 3.801 |
| 400 | 377.1 ± 17.86 | 94.28 | 4.736 |
| 500 | 443.7 ± 26.99 | 88.74 | 6.083 |
| 1000 | 879.4 ± 35.03 | 87.94 | 3.983 |
| 2000 | 1809 ± 83.14 | 90.45 | 4.596 |
| 3000 | 2807 ± 164.0 | 93.57 | 5.843 |

* Standard deviation of six replicate determinations.
† Average of six replicate determinations.

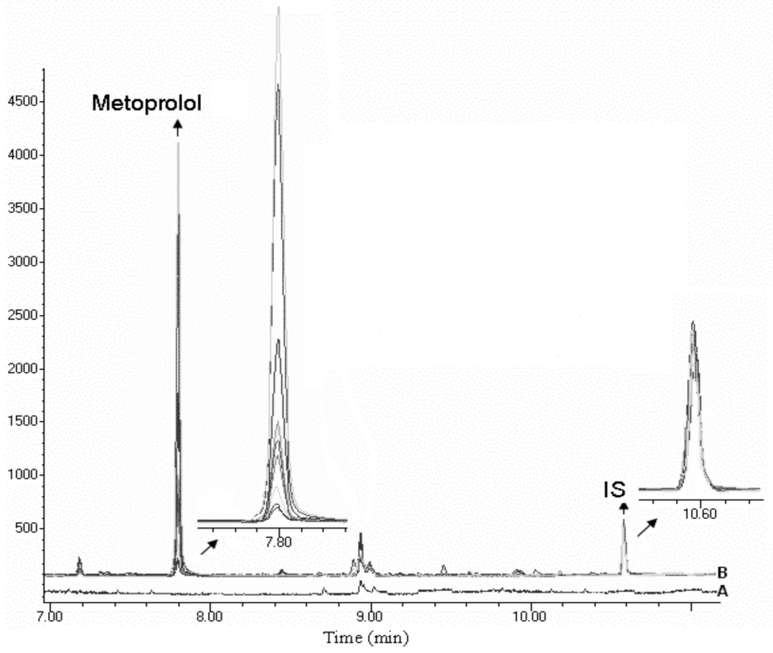


**Figure 2.** Typical SIM chromatogram of blank urine (A), urine spiked with metoprolol (50, 100, 200, 300, 400, 500, 1000, 2000, and 3000 ng/mL) and IS (500 ng/mL) (B).

analyzing three aliquots of each of the low and high concentration samples at room temperature for 8 h. Freeze-thaw stability (–20°C in urine) was checked through three cycles. Samples were stored at –20°C for 24 h and then thawed unassisted at room temperature. When completely thawed, 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 one week. The stability results indicated that no significant degradation of metoprolol in human urine was observed under the tested conditions (Table V).

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

| Samples | Conc. level (ng/mL) | A (mean ± SD)* | B (mean ± SD)† | % Matrix effect |
|---|---|---|---|---|
| Metoprolol | 150 | 143.5 ± 7.213 | 159.5 ± 4.945 | 89.97 |
| | 750 | 695.8 ± 11.43 | 737.3 ± 7.236 | 94.37 |
| | 3000 | 2856 ± 67.58 | 3057 ± 38.25 | 93.42 |
| IS | 500 | 455.1 ± 17.96 | 509.3 ± 12.34 | 89.36 |

* The amount of metoprolol and IS derivatized in blank urine sample's reconstituted solution (the final solution of blank urine after extraction and reconstitution).
† The amount of metoprolol and IS derivatized with MSTFA.

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

| Storage conditions | Concentration level (ng/mL) | Calculated conc. (ng/mL) | % RSD | % Relative error |
|---|---|---|---|---|
| Room temp. for 8 h | 500 | 487.8 | 7.821 | –2.44 |
| | 2500 | 2423 | 6.973 | –3.08 |
| Three freeze-thaw cycles | 500 | 476.2 | 5.834 | –4.76 |
| | 2500 | 2371 | 8.976 | –5.16 |
| 1 week at –20°C | 500 | 469.4 | 6.427 | –6.12 |
| | 2500 | 2403 | 8.438 | –3.88 |

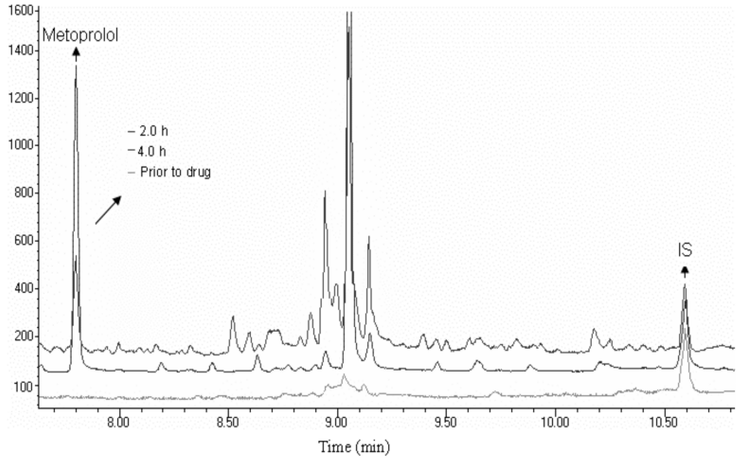


**Figure 3.** Typical SIM chromatogram of urine obtained from a patient at, before, and after oral administration of 100 mg metoprolol.

### Application of the method

The method was applied to the analysis of urine samples of a patient with hypertension treated with metoprolol. The clinical study protocol was approved by Ethics Committee of Faculty of Medicine at Ataturk University. The patient was a man who is 37 years old and 74 kg weight. The patient received an oral tablet (Problok) containing 100 mg of metoprolol. He had normal activity (standing or sitting) during the study but was never in a supine position during the 14 h following administration. Urine samples were collected at the following times (volume of urine): 0 (625 mL), 2 (500 mL), 4 (125 mL), 8 (300 mL), 10 (150 mL), and 14 (175 mL) h. The samples were immediately extracted and derivatized with MSTFA. Representative chromatograms obtained before and after administration of the metoprolol are shown in Figure 3. The urinary excretion data given in Figure 4 indicate that the GC–MS method is suitable for the assay of metoprolol after administration of metoprolol (Problok).

## Discussion

Kim et al. (3) have reported a GC–MS method after pre-column derivatization with α-methoxy-α-(trifluoromethyl)phenylacetyl chloride as a chiral derivatizing agent for the determination of metoprolol in human urine. Angier et al. (4) have reported the determination of metoprolol with other β-blockers in biological fluids by a GC–MS method after pre-column derivatization with pentafluoropropionic anhydride. Gowda et al. (14) have reported an LC–MS–MS method for the analysis of metoprolol in human plasma. The calibration curve of the LC–MS–MS method was linear for metoprolol in the range 5.0–500 ng/mL. Intra- and inter-day precision ranged from 4.82 to 8.42 and from 7.2 to 11.11% for metoprolol, respectively. The maximum recovery of metoprolol was 77.68%. The LOQ and LOD of method were found to be 5.0 and 1.0 ng/mL, respectively. Detection using LC–MS–MS would be a more sensitive approach but is costly and not yet available for every laboratory. Albers et al. (6) have reported an HPLC method with fluorescence detection for the analysis of metoprolol in human plasma. The calibration curve of the method was linear for metoprolol in the range 2.4–195.2 ng/mL. Precision throughout the whole working range was between 0.6–15.5%. Metoprolol recovery was determined at 73.0 ± 20.5, and the LOQ was 2.4 ng/mL.

Today, GC–MS is a powerful technique for highly specific and quantitative measurements of low levels of analytes in biological samples. As compared to HPLC, high-resolution capillary GC has been less frequently used because it reguires preconversion of multifunctional β-blockers into thermally stable volatile derivatives. However, it has inherently high resolving power and high sensitivity with excellent precision and accuracy, which allows simultaneous detection of expected and unexpected β-blockers, their metabolites, and contaminants. Also, the detection limits were lowered to pg levels by GC–MS in SIM mode (18)

The specificity of GC–MS method has been demonstrated by the representative chromatograms for metoprolol in human urine (Figures 2–3). The retention time of metoprolol in human urine is 7.8 min. The urine samples received from different persons have also been tested and showed no significant interference at the retention times of compounds of metoprolol. The recovery of metoprolol was achieved by developed liquid–liquid extraction procedure in human urine. Metoprolol was extracted from human urine with a mixture of ethylacetate and diethylether solvents. This solvent mixture gave an excellent recovery. The analytical recovery of metoprolol from human urine averaged 90.88%. Intra- and inter-day precision values for metoprolol in human urine were less than 6.0%. Metoprolol was extracted from urine with a solid-phase extraction procedure by Chiu et al. (8). This method is also the most comprehensive method which can extract metoprolol in a single extraction procedure.

In statistical comparison ($p > 0.05$) with other methods in the literature (3,6–8,12,14) the proposed method has indicated high accuracy, precision, and recovery.

Metoprolol was analyzed in plasma, amniotic fluid, and capillary blood by GC–MS, according to Ervik et al. (5). The minimum determinable concentration at a standard deviation of 10% was 1 nmol/L. The present method has the following advantage over the reported method (19). The LOQ of the reported method was 15.7 ng/mL, whereas the present method LOQ was 15 ng/mL.

Additionally, this method was applied to a patient with hypertension who had been given an oral tablet of 100 mg metoprolol. The amount of metoprolol was determined between 0–14 h in human urine. Unchanged metoprolol amount was accounted 4.39%. Our results for metoprolol excretion rates in human urine were similar to the findings previously reported (3).

## Conclusion

In the present work, a simple and sensitive GC–MS method has been developed for the determination of metoprolol in human urine. Also, the method was completely validated by using sensitivity, stability, specificity, linearity, accuracy, and precision parameters for the determination of metoprolol in human urine. Additional advantages of this method include small sample volume (0.5 mL), good extraction recovery from urine, and a readily available IS. Also, the extraction and derivatization procedures in this study were simple. Therefore, the proposed method can be used as a therapeutic drug-monitoring method in clinic to check the urine concentration of metoprolol in the patients with hypertension.

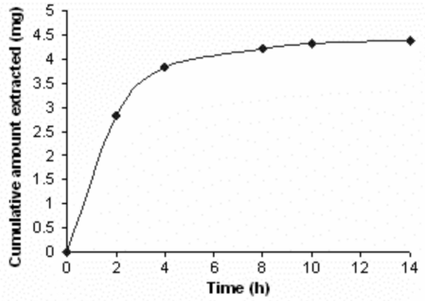


**Figure 4.** Cumulative urinary extraction of metoprolol following a single oral dose of 100 mg metoprolol (Problok).

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