Research Article

A New Liquid Chromatography-Tandem Mass Spectrometry Method for Determination of Bisoprolol in Human Plasma Samples

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Liquid chromatography (LC) coupled with mass spectrometry (MS) detection is one of the most powerful analytical tools for organic compound analysis. The advantages of using LC/MS methods over HPLC methods include: selectivity, chromatographic integrity, peak assignment, structural information, and rapid method development. In this paper, a new liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the determination of bisoprolol in human plasma samples, using metoprolol as internal standard and liquid-liquid extraction procedure. The assay has proven to be sensitive, specific and reproducible, suitable to determine the bisoprolol concentration, following a single oral administration of a 10 mg bisoprolol tablet in 22 healthy volunteers, in the bioequivalence study of Bisoprolol 10 mg coated tablets, produced by Antibiotice S.A. versus Concor 10 mg, produced by Merck.

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1. Introduction

Bisoprolol fumarate is a synthetic cardioselective β1-adrenergic blocker. Chemically, bisoprolol fumarate is (±)-1-[[2-(1-methylethoxy)ethoxy]methyl]phenoxy]-3-[(1-methylethyl)amino]-2-propanol(E)-2-butenedioate (2 : 1) [1]. It possesses an asymmetric carbon atom in its structure and is provided as a racemic mixture. The S(−) enantiomer is responsible for most of the beta-blocking activity.

The objectives of this work were to develop and to validate a simple, accurate, rapid and economic LC-tandem mass spectrometry method for the determination of bisoprolol in human plasma samples, using liquid-liquid extraction, and to present some of this method applications.

2. Materials and Methods

2.1. Instruments. All analyses were performed using the Agilent 1100 LC/MSD Trap XCT system. The system components included the Agilent 1100 Degasser, Agilent 1100 Binary Pump, Agilent 1100 Autosampler, Agilent 1100 Mass Selective Detector. The Bruker Daltonik software was used for system control and data acquisition. An analytical balance Mettler-Toledo XP56, a Sigma 2–16 K centrifuge and a Vibramax 110 shaker were used for the sample preparation.

The separation was performed using a reverse phase column (Zorbax SB-C18 Solvent Saver Plus, 3 × 100 mm, 3.5 μm, supplied by Agilent, USA).

2.2. Reagents. All solvents and other chemicals (acetonitrile, methanol, sodium hydroxide, tert-butyl methyl ether, water, formic acid) were HPLC grade provided by Merck’s Chemical Co., Darmstadt, Germany. The reference substances of bisoprolol and metoprolol (internal standard) were supplied from the USP Pharmacopoeia. The human plasma was obtained from Center for Blood Drawing and Preservation, Iasi, Romania.
2.3. Bisoprolol Stock Solution. Bisoprolol was dissolved in methanol, obtaining a bisoprolol stock solution of 500 μg/mL.

2.4. Metoprolol Stock Solution. The internal standard, metoprolol, was dissolved in methanol, obtaining a metoprolol stock solution of 500 μg/mL.

2.5. Solutions for Linearity Response. Eight bisoprolol concentrations were prepared in human plasma, covering the expected range of observed concentrations (1–100 ng/mL). The theoretical concentrations of bisoprolol calibration standards were 1.0, 2.0, 10.0, 20.0, 40.0, 60.0, 80.0, and 100.0 ng/mL.

2.6. Quality Control Samples. Plasma samples having bisoprolol theoretical concentrations of 3 ng/mL, 25 ng/mL and 75 ng/mL were considered to be appropriate to be used to validate the bioanalytical method.

2.7. Samples for Recovery. In order to determine the analyte and the internal standard recovery from the plasma, water samples containing the same bisoprolol concentrations as the quality control samples were prepared.

2.8. Samples Preparation. After alkalization with sodium hydroxide and addition internal standard solution, the 0.250 mL plasma sample was extracted with 2 mL tert-butyl methyl ether. The solvent was evaporated using a flow air at 40 °C. The solid residue was dissolved in a 0.250 mL mixture 0.1% formic acid solution—acetonitrile (50-50, v/v).

3. Results and Discussions

3.1. The Development of the LC-Tandem Mass Spectrometry Method. LC separation of bisoprolol and internal standard, metoprolol, has been carried out using the mobile phases consisting of different aqueous solutions and methanol or acetonitrile as organic phase. Hernando et al. [2] used a acetonitrile as organic mobile phase to lead to shorter retention times and better resolution of the bisoprolol and internal standard. Formic acid solution as additive in water was used by Li et al. [3] to improve the sensitivity of MS detection.

The LC-MS/MS method for determination of bisoprolol in human plasma samples described in this paper was performed using a mobile phase consist in mixture 0.1% formic acid solution (pH 3)—acetonitrile (50-50, v/v). The LC system was operated at 0.3 mL/min, using the binary pump. The column temperature was 40°C. The injection volume was 5 μL and represented no more 5% of the total sample available for injection. Short run times of about 3 minutes were achieved for both bisoprolol and internal standard, metoprolol. Bisoprolol was eluted at 1.7 minutes and metoprolol at 1.9 minutes. The peaks of interest were free from interfering peaks at their respective retention time.

To minimize undesirable fragmentation voltages were tested from 80 to 200 V. At 100 V, the MS response of bisoprolol and metoprolol showed both minimal undesirable fragmentation and highest response.

The mass transition ion-pair was followed as m/z 326.2 → 116.1 for bisoprolol and m/z 268.2 → 191.0, for metoprolol, as sustained Bhatt et al. [4].

The protonated molecular ion of bisoprolol [M+H]+ (m/z 326.3) was tested to give the highest sensitivity. Based on the optimization results, m/z 116.2 was selected as the quantifier ion. Also, the protonated molecular ion of metoprolol [M+H]+ (m/z 268) was tested and m/z 116.2 was selected as the quantifier ion. We have chosen products ions with the same m/z value for bisoprolol and metoprolol, respectively, because a possible suppression effect would influence the quantification of both the analyte and internal standard in the same way.

Other mass spectrometric parameters (gas temperature, gas pressure and gas flow) were adjusted to get a maximum signal for bisoprolol. The nebulizing gas flow rate was set at 101/min, drying gas temperature at 350 °C, and the capillary voltage at 4000 V. The response of bisoprolol and metoprolol were measured by MRM in the positive ionization mode with a collision energy of 20 V.

3.2. Validation of the LC-Tandem Mass Spectrometry Method. The method was validated according Guidance for Industry: Bioanalytical Method Validation [5].

The parameters usually examined in the validation process are selectivity/specificity, linearity, limit of quantification, accuracy and precision, stability.

3.2.1. Selectivity. The reversed-phase HPLC method described in this paper has been tested for possible interferences from other plasma factors. Plasma aliquots from six different sources were assessed for analysis in order to investigate the plasma components behavior.

As it can be seen in Figure 1, no overlapping peaks were detected at bisoprolol and internal standard retention time, 1.7 minutes and 1.9 minutes, respectively. The bioanalytical method proved to be selective.

3.2.2. Linearity and Lower Limit of Quantification. The linearity was investigated for a bisoprolol theoretical concentration range between 1 ng/mL and 100 ng/mL and the calibration curve was derived by plotting the peak-height ratios of the analyte and the internal standard against the concentration of bisoprolol, using linear regression analysis.

The least-square linear regression revealed that the relationship was linear in the investigated domain, with a correlation coefficient of 0.998599, meeting the acceptance criteria (r² ≥ 0.990), as it can be seen in Figure 2.

The lower limit of quantification, that is, the lowest standard level with a coefficient of variation less than 20%, is for bisoprolol 0.990 ng/mL with 41.433 signal to noise ratio. The bioanalytical method proved to be sensitive, allowing a precise quantification of concentrations as low as 1 ng/mL (see Figure 3). Results are presented in Table 1.
3.2.3. Accuracy and Precision. Accuracy of the analytical method represents the degree of closeness of the determined values of an analyte to the nominal/or known true value declared from an individual sample. The accuracy of a bioanalytical method is expressed as a percentage of the nominal value (% nominal).

Precision of the analytical method represents the degree of dispersal of the values determined of an analyte, from a series of samples processed and analyzed individually from a homogeneous volume of biological matrix. Precision of a bioanalytical method is expressed as the coefficient of variation of the concerned series of determinations, CV (%).

The accuracy and precision of this method were calculated for three concentrations of bisoprolol in human plasma. Six replicate samples having bisoprolol theoretical concentrations of 3 ng/mL (QC1), 25 ng/mL (QC2) and 75 ng/mL (QC3) were injected into the system. Table 2 summarizes the results obtained for the intraday parameters.

The interday precision and accuracy was evaluated also using
Table 1: Lower limit of quantification.

| Analyte concentration (ng/mL) | Conc. (ng/mL) | % nominal | Signal/noise ratio |
|-------------------------------|---------------|-----------|-------------------|
| 0.989                         | 99.945        |           | 44.500            |
| 1.407                         | 142.094       |           | 36.000            |
| 0.958                         | 96.802        |           | 46.100            |
| 1.167                         | 117.863       |           | 31.700            |
| 1.175                         | 118.723       |           | 41.500            |
| 1.241                         | 125.400       |           | 48.800            |
| N                             | 6             | 116.804   | 41.433            |
| Mean                          | 1.156         |           | 41.433            |
| SD (±)                        | 0.166         |           |                   |
| CV(%)                         | 14.339        |           |                   |

**Acceptance criteria**

4 out of 6 LLQC must be 100±20% nominal value.
Mean % nominal 100 ± 20%
CV (%) ≤ 20%
Signal/noise ratio ≥ 5

Table 2: Evaluation of intraday precision and accuracy for bisoprolol spiked quality control samples.

| C<sub>th</sub> = 3 ng/mL | C<sub>th</sub> = 25 ng/mL | C<sub>th</sub> = 75 ng/mL |
|---------------------------|---------------------------|---------------------------|
| C<sub>exp</sub> (ng/mL)    | % nominal                 | C<sub>exp</sub> (ng/mL)   | % nominal | C<sub>exp</sub> (ng/mL) | % nominal |
| (1) 2.912                 | 98.391                    | 22.524                    | 91.263    | 69.203                  | 93.468    |
| (2) 3.003                 | 101.439                   | 24.862                    | 100.737   | 68.525                  | 92.552    |
| (3) 3.262                 | 110.192                   | 23.762                    | 96.281    | 66.781                  | 90.196    |
| (4) 2.829                 | 95.568                    | 25.189                    | 102.062   | 76.691                  | 103.580   |
| (5) 3.018                 | 101.960                   | 21.739                    | 88.085    | 65.844                  | 88.931    |
| (6) 2.854                 | 96.431                    | 21.371                    | 86.593    | 74.009                  | 99.958    |
| Mean                      | 2.980                     | 100.664                   | 23.241    | 94.170                  | 70.176    | 94.781    |
| SD                         | 0.158                     | 1.610                     | 4.268     |                       |           |
| CV %                       | 5.296                     | 6.927                     | 6.082     |                       |           |

**Acceptance criteria**

67% Total QCs must be 100 ± 15% nominal values
50% QCs per level must be 100 ± 15% nominal values
Mean % nominal 100 ± 15%
CV (%) ≤ 15%

C<sub>th</sub> = theoretical concentration. C<sub>exp</sub> = experimental concentration. SD = standard deviation. CV % = coefficient of variation

six aliquots for each quality control sample concentration, prepared and analysed in six different days. The results are presented in Table 3.

Intra-and interday precision of analysis was <8% and accuracy range was from 94.170% to 102.540%.

The values for the investigated parameters proved to be lower than the one reported by Oniscu et al. [6], employing a HPLC method with fluorescence detection. Also, Liu et al. [7] reported an accuracy ranged from 89.4%–113%, employing a precipitation with acetonitrile procedure for plasma sample preparations.

3.2.4. Recovery. Recovery of Bisoprolol was evaluated by comparing analyte response of six extracted samples of low, medium, and high quality control samples to those of six appropriately diluted standard solutions. Mean recovery values for Bisoprolol are 76.529, 78.479, and 79.863% at low, medium and high quality control levels, respectively.

For internal standard, mean internal standard response of eighteen extracted samples was compared to the mean internal standard responses of eighteen appropriately diluted internal standard solutions. Mean recovery value for the internal standard is 90.568%.

3.2.5. Stability Tests. To test stability, a series of standards samples was prepared from freshly made stock solutions in the same solvent used for the assay. The lowest and highest concentration of the quality control (3 ng/mL and
75 ng/mL), including the analyte and internal standard (when appropriate) were used. Human plasma samples of each concentration were prepared in enough volume to have multiple aliquots. The aliquots of each concentration were processed and quantified immediately in order to provide the reference (fresh) values and other six aliquots of each concentration were processed for the desired tests.

The following subsections present the procedure carried out and the corresponding results.

(a) Stability of the Analyte after Sample Processing at Room Temperature. Samples prepared at low (QC1) and high (QC3) quality control levels were submitted to the extraction procedure and kept at room temperature under ambient laboratory conditions (stability samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed and analyzed with 6 replicates of stability samples in a single run. Concentrations were calculated to determine % change over time.

### Table 3: Evaluation of interday precision and accuracy for bisoprolol spiked quality control samples.

| C_{th} (ng/mL) | C_{exp} (ng/mL) | Accuracy (%) | C_{exp} (ng/mL) | Accuracy (%) | C_{exp} (ng/mL) | Accuracy (%) |
|---------------|----------------|--------------|----------------|--------------|----------------|--------------|
| (1) 3.015     | 101.860        | 24.331       | 98.586         | 70.921       | 95.788         |
| (2) 2.879     | 97.276         | 23.332       | 94.539         | 77.073       | 104.097        |
| (3) 3.257     | 110.048        | 23.109       | 93.636         | 77.932       | 105.256        |
| (4) 2.964     | 100.136        | 24.013       | 97.297         | 70.590       | 95.340         |
| (5) 2.943     | 99.439         | 23.109       | 93.636         | 77.932       | 105.256        |
| (6) 3.152     | 106.480        | 24.910       | 100.933        | 74.865       | 101.115        |
| Mean          | 3.035          | 102.540      | 24.684         | 100.018      | 74.494         | 100.613      |
| SD            | 0.142          | 102.540      | 3.092          | 1.940        | 3.092          | 4.151        |
| CV %          | 4.686          | 102.540      | 13.314         | 7.860        | 13.314         | 13.704       |

**Acceptance criteria**

67% Total QCs must be 100 ± 15% nominal values
50% QCs per level must be 100 ± 15% nominal values
Mean % nominal 100 ± 15%
CV (%) ≤ 15%

### Table 4: Stability of analyte following sample processing at room temperature.

| Analyte: Bisoprolol | Biological matrix: Human Plasma | Storage condition: 31 Hours at Room Temperature |
|---------------------|--------------------------------|-----------------------------------------------|
| Sample              | QC1 (3 ng/mL) | Stability Samples | QC3 (75 ng/mL) | Stability Samples |
| Conc.               | Measured | % nominal | Measured | Stability Samples | Measured | % nominal | Measured | Stability Samples |
| 2.682               | 2.838    | 90.611    | 80.224   | 108.352          | 82.798   |
| 2.330               | 2.639    | 78.719    | 82.755   | 111.771          | 63.262   |
| 3.082               | 2.434    | 104.134   | 78.821   | 106.457          | 75.379   |
| 2.380               | 2.079    | 80.392    | 79.826   | 107.815          | 80.616   |
| 2.117               | 2.047    | 71.504    | 91.323   | 123.343          | 58.511   |
| 2.619               | 2.745    | 88.475    | 84.105   | 113.595          | 79.410   |
| N                   | 6       | 6        | 6       | 6                 | 6       |
| Mean                | 6.454   | 85.639   | 82.842   | 111.889          | 73.329   |
| SD (±)              | 0.338   | 3.338    | 4.597    | 10.049           | 13.704   |
| CV (%)              | 13.314  | 13.314   | 5.549    | 13.704           | 13.704   |
| % Change            | −2.808  | −11.483  | −11.483  |                   |          |

**Acceptance criteria**

67% comparison samples must be 100 ± 15% nominal values
Mean % nominal of comparison samples 100 ± 15%
CV (%) ≤ 15%
% Change ± 15%
Table 5: Stability of analyte in biological matrix at room temperature.

| Analyte: Bisoprolol | Biological matrix: Human plasma | Storage condition: 4 Hours at Room Temperature |
|---------------------|---------------------------------|---------------------------------------------|
| QC1 (3 ng/mL)       | Stability Samples               | QC3 (75 ng/mL)                              |
| Comparison Samples  | Measured Conc. % nominal        | Stability Samples Measured Conc. % nominal  |
| Measured Conc.      | 3.509 118.561                   | 3.540 61.764                                |
|                      | 2.839 95.898                    | 3.502 74.312                                |
|                      | 3.453 116.639                   | 3.281 73.697                                |
|                      | 3.187 107.684                   | 3.514 72.186                                |
|                      | 4.310 145.604                   | 3.616 68.557                                |
|                      | 3.123 105.510                   | 3.688 75.832                                |
| N                   | 6                               | 6                                           |
| Mean                | 3.403 114.983                   | 3.523 71.058                                |
| SD (±)              | 0.506                           | 0.138                                       |
| CV(%)               | 14.864                          | 3.925                                       |
| % Change            | 3.522                           | 3.566                                       |

Acceptance criteria:
67% comparison samples must be 100 ± 15% nominal values
Mean % nominal of comparison samples 100 ± 15%
CV (%) ≤ 15%
% Change ± 15%

Table 6: Stability of analyte in biological matrix after 3 freeze-thaw cycles at −25 ± 10°C.

| Analyte: Bisoprolol | Biological matrix: Human plasma | Storage condition: −25 ± 10°C |
|---------------------|---------------------------------|--------------------------------|
| QC1 (3 ng/mL)       | Stability Samples               | QC3 (75 ng/mL)                              |
| Comparison Samples  | Measured Conc. % nominal        | Stability Samples Measured Conc. % nominal  |
| Measured Conc.      | 3.015 101.860                   | 2.934 70.921                                |
|                      | 2.879 97.276                    | 3.239 77.073                                |
|                      | 3.257 110.048                   | 3.096 77.932                                |
|                      | 2.964 100.136                   | 3.180 70.590                                |
|                      | 2.943 99.439                    | 3.049 75.582                                |
|                      | 3.152 106.480                   | 3.063 74.865                                |
| N                   | 6                               | 6                                           |
| Mean                | 3.035 102.540                   | 3.093 74.494                                |
| SD (±)              | 0.142                           | 0.107                                       |
| CV(%)               | 4.686                           | 3.454                                       |
| % Change            | 1.920                           | 6.212                                       |

Acceptance criteria:
67% comparison samples must be 100 ± 15% nominal values
Mean % nominal of comparison samples 100 ± 15%
CV (%) ≤ 15%
% Change ± 15%
Bisoprolol is found to be stable for 31 hours at room temperature under ambient laboratory conditions after sample processing with % changes (ratio between mean concentration of stability samples and mean concentration of comparison samples) of $-11.483$ and $-2.808\%$. Results are presented in Table 4.

(b) Stability of Analyte in Biological Matrix at Room Temperature. Samples were prepared at low (QC1) and high (QC3) quality control levels, aliquoted and frozen at $-25 \pm 10^\circ C$. Some of the aliquots of quality control samples were subjected to three freeze-thaw cycles (stability samples). The remaining aliquots were not thawed (comparison samples). A calibration curve and 6 replicates of low and high quality control samples (comparison samples) were freshly processed with 6 replicates of stability samples and analyzed in a single run. Concentrations were calculated to determine % change over freeze-thaw cycles. Bisoprolol is found to be stable in human plasma after three freeze-thaw cycles with % changes of 1.920 and 6.212%. Results are presented in Table 6.

3.3. The Applications of LC-Tandem Mass Spectrometry Method. The assay has proven to be suitable to determine the bisoprolol concentration in the bioequivalence study of Bisoprolol 10 mg coated tablets produced by Antibiotice S.A. (referred to as test drug) versus Concor® 10 mg coated tablets produced by Merck (referred to as reference drug). In Figure 4, average bisoprolol concentrations recorded for 22 volunteers are plotted against time for both test and reference drugs. The concentration profiles are similarly, fitting the results obtained for the in vitro dissolution test (see Figure 5).

Based on the determined bisoprolol concentrations, the calculated pharmacokinetic parameters demonstrated that the drug produced by Antibiotice S.A. is bioequivalent with the one produced by Merck.

4. Conclusions

LC-tandem mass spectrometry method described and validated above is sensitive, accurate, precise, rapid, and efficient. The developed method can be applied for the determination of bisoprolol from human plasma samples (e.g., for pharmacokinetic parameters).

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