Experimental part

Materials and methods

A number of 20 healthy volunteers were enrolled in the study. The study was conducted according to principles of Declaration of Helsinki (1964) and its amendments (Tokyo 1975, Venice 1983, Hong Kong 1989). The clinical protocol was reviewed and approved by the Ethics Committee of the University of Medicine and Pharmacy Iuliu Hatieganu, Cluj-Napoca.

Study protocol

After an overnight fast the volunteers received a single 5 mg nebivolol dose (Nebilet 5 mg tablets, Berlin-Chemie AG, Germany) at 8:00 a.m. along with 150 mL of water. Blood samples (5 mL) were taken via an indwelling venous cannula according to the following time schedule: before drug administration (0 h), and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, 24, 36 and 48 h after drug administration. Within 10 minutes of collection, blood samples were centrifuged at 5000 rpm for 10 min and plasma samples were stored at -20°C until analysis.

Drug analysis from plasma

Nebivolol and its corresponding hydroxylated metabolite (4-hydroxy-nebivolol) plasma concentrations were determined by a validated liquid chromatography–mass spectrometry method [11]. The HPLC system was an Agilent 1100 series (binary pump, autosampler, thermostat temperature was set at 48°C. The mass spectrometry method was used. The mobile phase consisted of 0.2% (V/V) formic acid in water and acetonitrile, and gradient elution was as follows: start with 10% acetonitrile, at 6.6 min 43% acetonitrile. The flow rate was 1 mL/min, and the thermostat temperature was set at 48°C. The mass spectrometry detection was in multiple reaction monitoring mode, positive ions, using an electrospray ionization source. The ion transitions monitored were m/z 406 for nebivolol (single ion monitoring mode) and m/z 404 from m/z 422 for its hydroxylated metabolite (multiple ion monitoring mode). The sample preparation was

Keywords: nebivolol, 4-hydroxy-nebivolol, compartmental pharmacokinetic analysis

Nebivolol is a third generation, highly-selective beta1-blocker, also having a vasodilatatory effect due to enhancing nitric oxide bioavailability via the L-arginine-nitric oxide pathway [1-3]. This unique characteristic among beta-blockers can offer a supplementary benefit in hypertension treatment besides lowering the blood pressure per se, being well-known the protective action of nitric oxide against cardio-vascular risk factors, mainly atherosclerosis [4, 5]. At therapeutic doses (5 mg once a day for adults), nebivolol is considered a safe drug, with a relative low incidence of adverse reactions. Despite its therapeutic benefit, nebivolol has some important side effects that arise in intensity in relation with the drug plasma levels. The most important adverse reactions are paraesthesia, bradycardia, AV block, acute cardiac failure, hypotension, dizziness, fatigue, edema or headache [6, 7].

Pharmacokinetics, by the quantitative study of the processes that take place depending on time [8, 9], during the stages of absorption, distribution, metabolism and excretion of a drug, offers a better understanding of the relationship between the given/administered dose and the pharmacological effect [10]. By using compartmental and non-compartmental analysis, the corresponding pharmacokinetic parameters of the drug can be obtained, and it can be used in drug formulation, bioequivalence or in therapeutic drug monitoring for patient-specific dose adjustment.

The aim of this study was to create and to use a pharmacokinetic model that can accurately describe the kinetic processes involved in absorption, distribution, metabolism and elimination of nebivolol and 4-hydroxy-nebivolol after oral administration of a single dose of nebivolol in healthy volunteers.
Table 1

<table>
<thead>
<tr>
<th>Model</th>
<th>Presystemic metabolism</th>
<th>Systemic metabolism kinetics</th>
<th>Nebivolol, Number of compartments</th>
<th>4-hydroxy-nebivolol, Number of compartments</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>No</td>
<td>1st order</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M2</td>
<td>No</td>
<td>1st order</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>M3</td>
<td>No</td>
<td>1st order</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>M4</td>
<td>Yes, 1st order kinetics</td>
<td>1st order</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M5</td>
<td>Yes, 1st order kinetics</td>
<td>1st order</td>
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<td>1</td>
</tr>
<tr>
<td>M6</td>
<td>Yes, 1st order kinetics</td>
<td>1st order</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

performed by adding 0.6 mL methanol to 0.2 mL plasma in an Eppendorf tube. The tube was vortexed for 10 s, then centrifuged for 6 min at 7425 g. The supernatant was transferred to an autosampler vial, and 40 µL was injected into the chromatographic system. The calibration curves of nebivolol and its active metabolite (4-hydroxy-nebivolol) were linear at a concentration range of 0.1–16 ng/mL plasma.

Pharmacokinetic analysis

The compartmental pharmacokinetic analysis was employed for analysis of plasma versus time levels of nebivolol and its metabolite for each individual dataset obtained from the volunteers (20 datasets).

Six mathematical models were created in order to analyse the pharmacokinetics of nebivolol and its active metabolite (table 1).

The differences between the models consisted in assumptions about the existence of presystemic metabolism of nebivolol and about the number of compartments for both nebivolol and 4-hydroxy-nebivolol.

For example, the first pharmacokinetic model employs no presystemic metabolism of nebivolol and mono-compartmental distribution for both nebivolol and its metabolite. The model MC6 employs existence of presystemic metabolism and biphasic distribution for both nebivolol and 4-hydroxy-nebivolol. Each model considers that the elimination of compounds is a 1st order kinetic process, as well as the systemic metabolism of nebivolol to 4-hydroxy-nebivolol.

The schematic representation of the kinetic processes from model M6 are presented in figure 1.

For each kinetic model, the corresponding mathematical differential equations were written and run by using Phoenix 6.1 software package (Certara, SUA). The equations of model M6 are presented in figure 2.

![Fig. 1. Schematic representation of kinetic processes from model M6, where 0 is absorption compartment of nebivolol; 1 and 2 are central compartments of nebivolol and 4-hydroxy-nebivolol; 3 and 4 are their corresponding peripheral distribution compartments, tlag is the latency time for absorption; k01 is the absorption rate constant of nebivolol, f is the fraction of nebivolol converted into metabolite during absorption (presystemic metabolism); k13, k31, k24, k42 are the distribution rate constants; k10 and k20 are the elimination rate constants for nebivolol (non-metabolic) and 4-hydroxy-nebivolol](image1)

![Fig. 2. The mathematical equations of the kinetic model M6, where \( Q_N \) and \( Q_M \) are the quantities of nebivolol in central and peripheral compartment respectively; \( Q_{MC} \) and \( Q_{MP} \) are the quantities of metabolite in central and peripheral compartments; \( Conc_N \) and \( Conc_M \) are the plasma concentrations of nebivolol and 4-hydroxy-nebivolol, \( V_F \) is the apparent volume of distribution of the central compartment. All the other parameters used were previously presented](image2)
Results and discussions

The mean plasma concentrations of nebivolol and 4-hydroxy-nebivolol were analysed using the six kinetic models previously presented, after their implementation in Phoenix software. The same settings of the software minimisation engine were used for all analysed models: weighting scheme 1/y (1/observed concentration), minimisation method: Gauss-Newton (Levenberg and Hartley variant), convergence criterion: 0.0001.

The Akaike index (automatically calculated and provided by the analysis software) was used for model discrimination [12]. The model that fit better to the data is characterised by a smaller Akaike index. The Akaike values for the six analysed models are presented in figure 3.

By analysing the Akaike values presented in figure 3, one can see that the model M6 fits the data better than its concurrent models, having the smallest Akaike value, so it was chosen as representative for describing the kinetics of nebivolol and 4-hydroxy-nebivolol after oral administration of a single dose of nebivolol.

In figure 4 is presented a typical fitting of a subject data to representative model M6 in comparison with M1. There is a better correlation between the experimental (observed) and the fitted (predicted) values for nebivolol and 4–hydroxy-nebivolol concentrations in case of the model M6 than in case of the model M1.

According to the M6, the pharmacokinetics of nebivolol is characterised by a first order absorption rate kinetics with presystemic metabolism to 4–hydroxy-nebivolol. Both nebivolol and 4-hydroxy-nebivolol are characterised by bicompartimental distribution. After absorption, nebivolol is converted to 4-hydroxy-nebivolol by systemic metabolism, following a first-order kinetic process. Both compounds are further eliminated from the body by first order kinetic processes. By using this representative pharmacokinetic model for nebivolol and 4–hydroxy-nebivolol, their characteristic pharmacokinetic parameters were calculated (table 2).

A large variability of calculated kinetic parameters of nebivolol and its metabolite can be observed between the 20 subjects participating in the study (table 2). However, this is commonly seen in such studies due to natural biologic and physiologic differences between subjects (inter-subject variability) [13, 14].

The absorption of nebivolol is delayed for about 0.30±0.23 h after oral administration, the time needed for drug to reach into duodenum. The absorption rate constant is 2.60±2.72 h⁻¹, that means an absorption half-life of about 0.266 h. During absorption, about 10% of the bioavailable amount of nebivolol is converted to metabolite that appears in plasma.

The apparent volume of distribution for central compartment of both nebivolol and metabolite is about 1410±903 L, this high value being expected as both compounds are lipophilic and highly bounded on tissue proteins [1]. The kinetic model M6 considers for nebivolol two elimination paths: by systemic metabolism to 4–hydroxy-nebivolol (characterised by a rate constant k12) and by other processes (characterised by an overall rate constant k10). As can be observed from table 2, the value of k12 (0.318±0.208 h⁻¹) is much higher than k10 (5.53 ± 10⁻⁴ to 6.23 ± 10⁻⁴ h⁻¹). This means that a large majority of 99.8% from nebivolol is eliminated from the body by metabolism to 4–hydroxy-nebivolol, the rest being eliminated by metabolism to other metabolites or by direct renal excretion. Both nebivolol and its metabolite are distributed between central and peripheral compartments, the last having a greater affinity for each compound (k13>k31 and k24>k42).

The observed concentrations of 4–hydroxy-nebivolol are due to both presystemic and systemic biotransformation of nebivolol. The metabolite is eliminated following a first-order kinetic process, characterised by a rate constant of 0.332±0.305 h⁻¹.

Conclusions

In order to describe the kinetics of nebivolol and its metabolite 4-hydroxy-nebivolol after oral administration of a single oral dose of 5 mg nebivolol, six mathematical models were tested. These models involve differences
regarding the presystemic metabolisation of nebivolol to its metabolite and the mono- or bicompartimental distribution of the compounds.

After data analysis, the representative model for the pharmacokinetics of nebivolol and its metabolite was assessed. This model considers that nebivolol is absorbed following a first-order process and is partially converted during absorption to the metabolite. The kinetics of nebivolol is characterised by bicompartimental distribution and first order kinetic elimination processes (99.8% by biotranformation to metabolite, the rest by other paths). The metabolite 4-hydroxy-nebivolol has also a bicompartimental distribution and a first order elimination kinetics.

The knowledge of the drug kinetics in the body (e.g. the kinetic model) is the starting point for other important analysis like the prediction of drug plasma levels at other doses or when multiple doses are administered, pharmacokinetic population modelling or further mathematical correlations between drug kinetics and the pharmacological effect intensity.

Acknowledgement: This work was supported by CNCS Romania - project PN-II-IDPCE-2011-3-0731. PhD student Ana-Maria Gheldiu acknowledges financial support from a POSDRU grant, no. 1591.5/S/136893 with title: “Interuniversity strategic partnership in order to improve the quality of medical research in by granting doctoral and postdoctoral scholarships - DocMed.Net_2.0”.

References
2. KAMP, O., METRA, M., BUGATTI, S., et al., Nebivolol Haemodynamic Effects and Clinical Significance of Combined beta-Blockade and Nitric Oxide Release, Drugs, 70, nr. 1, 2010, p.: 41-56

Table 2
THE KINETIC PARAMETERS OF NEBIVOLOL AND 4-HYDROXY-NEBIVOLOL CALCULATED WITH MODEL M6

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Mean</th>
<th>SD</th>
<th>CV%</th>
<th>Median</th>
<th>Geometric Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tlag (hr)</td>
<td>0.301</td>
<td>0.237</td>
<td>79.0</td>
<td>0.338</td>
<td>0.0979</td>
</tr>
<tr>
<td>k01 (hr⁻¹)</td>
<td>2.60</td>
<td>2.72</td>
<td>105</td>
<td>1.51</td>
<td>1.50</td>
</tr>
<tr>
<td>f</td>
<td>0.106</td>
<td>0.0739</td>
<td>69.5</td>
<td>0.0977</td>
<td>0.0827</td>
</tr>
<tr>
<td>k10 (hr⁻¹)</td>
<td>5.53*10⁻⁴</td>
<td>6.23*10⁻⁴</td>
<td>113</td>
<td>1.86*10⁻⁴</td>
<td>3.37*10⁻⁴</td>
</tr>
<tr>
<td>k12 (hr⁻¹)</td>
<td>0.318</td>
<td>0.208</td>
<td>65.6</td>
<td>0.291</td>
<td>0.233</td>
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<tr>
<td>k13 (hr⁻¹)</td>
<td>1.72</td>
<td>3.11</td>
<td>181</td>
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<tr>
<td>k21 (hr⁻¹)</td>
<td>1.58</td>
<td>2.44</td>
<td>155</td>
<td>0.397</td>
<td>0.559</td>
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<tr>
<td>k20 (hr⁻¹)</td>
<td>0.332</td>
<td>0.305</td>
<td>92.0</td>
<td>0.296</td>
<td>0.128</td>
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<tr>
<td>k24 (hr⁻¹)</td>
<td>0.543</td>
<td>0.680</td>
<td>125</td>
<td>0.354</td>
<td>0.316</td>
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<tr>
<td>k42 (hr⁻¹)</td>
<td>0.136</td>
<td>0.152</td>
<td>112</td>
<td>0.0497</td>
<td>0.0495</td>
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<tr>
<td>V_F (L)</td>
<td>1410</td>
<td>903</td>
<td>63.9</td>
<td>1270</td>
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