Predicting Drug Metabolism: A Site of Metabolism Prediction Tool Applied to the Cytochrome P450 2C9

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Received November 20, 2002

The aim of the present study is to develop a method for predicting the site at which molecules will be metabolized by CYP 2C9 (cytochrome P450 2C9) using a previously reported protein homology model of the enzyme. Such a method would be of great help in designing new compounds with a better pharmacokinetic profile, or in designing prodrugs where the compound needs to be metabolized in order to become active. The methodology is based on a comparison between alignment-independent descriptors derived from GRID Molecular Interaction Fields for the CYP 2C9 active site, and a distance-based representation of the substrate. The predicted site of metabolism is reported as a ranking list of all the hydrogen atoms of each substrate molecule. Eighty-seven CYP 2C9-catalyzed oxidative reactions reported in the literature have been analyzed. In more than 90% of these cases, the hydrogen atom ranked at the first, second, or third position was the experimentally reported site of oxidation.

Introduction

The cytochrome P450s are a superfamily of enzymes responsible for the metabolism of many druglike molecules. Several properties of these enzymes, such as the rate and site of metabolism, inhibition, and induction and the selectivity for the various isoforms, need to be considered in the lead optimization process during the development of new therapeutic agents. The design of computational predictive methods for each of these properties is therefore of increasing interest for the drug industry. Computational models to predict cytochrome inhibition,[1−4] rate of metabolism,[5−10] site of metabolism,[6−11] and selective interaction analysis[12] have already been described.

Such predictions are challenging due to the many different isoforms that can be involved in the metabolic pathway of a single compound, the number of possible sites of metabolism for each isoform and the lack of structural information about human cytochromes.

The structure of mammalian cytochrome P450 2C5 from rabbit was recently reported.[13] This enzyme has a high degree of similarity (>82%) and identity (>77%) with the CYP 2C family in humans, but the crystal structure only has a relatively low resolution (3 Å).[1,12] It can nevertheless be used as a template in homology modeling for the CYP 2C enzymes. [Similar homology protein models were previously used to predict competitive inhibition constants[1] and to obtain structure−activity information in selectivity analysis[12]]. In addition, structural information about the different steps in the oxidative reaction of camphor catalyzed by the CYP P450 Cam was recently reported.[14] The authors described how certain residues in the binding site play an important role in the orientation of one particular hydrogen atom toward the oxygen atom attached to the heme moiety. Their study shows how the structure of the binding site influences the presentation of this hydrogen atom toward the Fe−O complex, and such information could be of great help both in designing new compounds with a better pharmacokinetic profile and in avoiding the presence of toxic metabolites, by chemically protecting metabolically labile moieties in drug candidates. One might also use metabolism site prediction to design of prodrugs where the compound needs to be metabolized in order to become active.

CYP 2C9 is one of the cytochromes involved in the first pass metabolism of drugs limiting their oral availability. For example, there are several CYP 2C9 substrates that belong to the nonsteroid antiinflammatory drug class such as diclofenac,[15,16] ibuprofen,[17] naproxen,[18] and piroxicam.[19] Moreover, progesterone is also metabolized by CYP2C9 as well as the anticoagulant compounds sharing a coumarin like substructure, i.e., *S*-warfarin.[20,21]

The aim of the present study is to develop a fast, easy to interpret, and computationally unexpensive method for predicting CYP 2C9 metabolism site using developed homology model from CYP 2C5 and the 3D structure of the metabolized substrates. It is not within the scope of this paper to predict the rate of metabolism, since other experimental information based on empirically determined data (K_m or/and V_{max}) would probably be needed to develop such a model.

Materials and Methods

The proposed methodology involves the calculation of one descriptor set for the CYP protein and another set of descriptors for each substrate. At the end of the procedure, the descriptors for the substrate and the protein are compared

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10.1021/jm021104i CCC: $25.00
Published on Web 05/10/2003

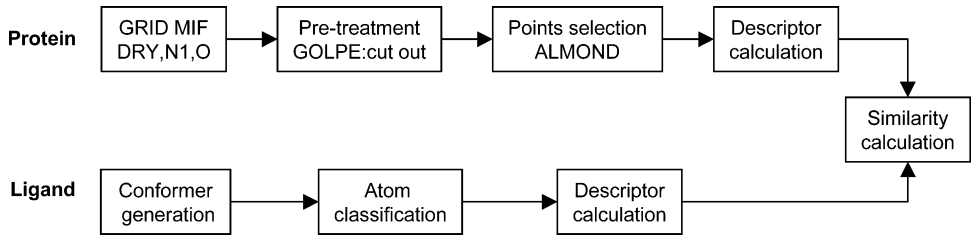


Figure 1. Procedure flowchart.

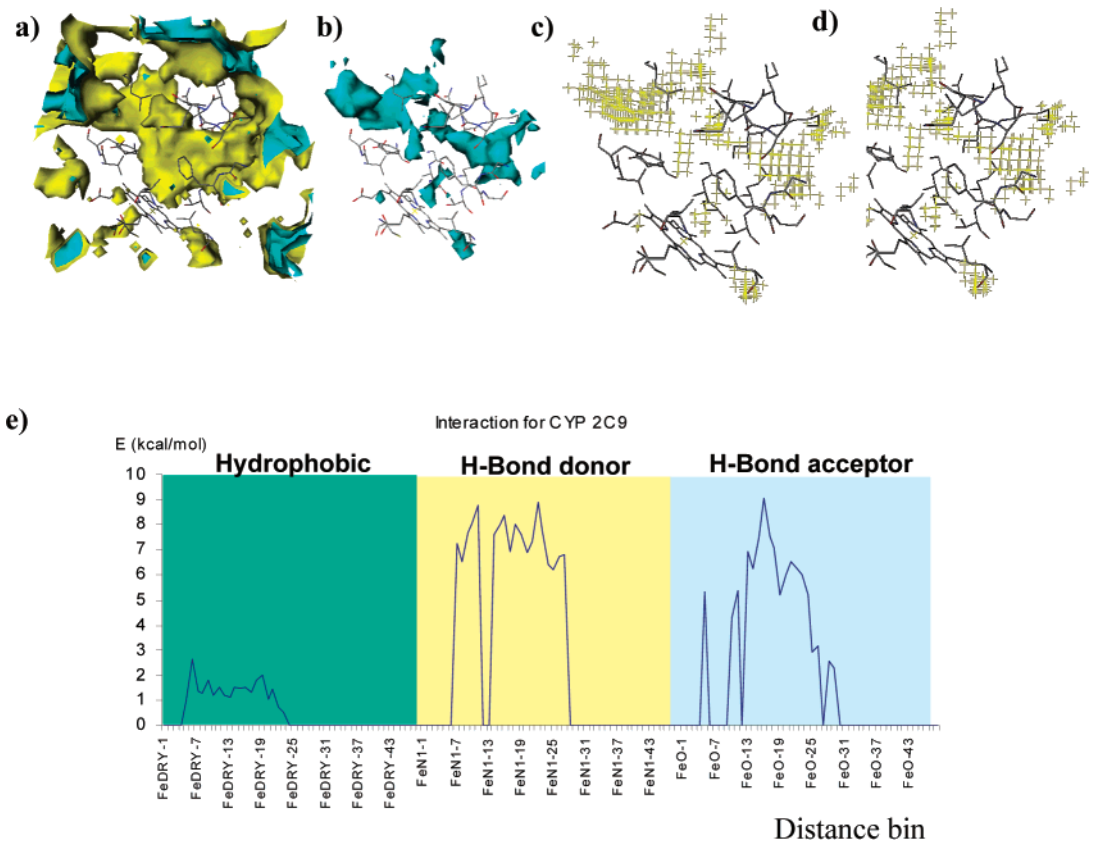


Figure 2. Protein treatment. (a) Molecular Interaction Field (MIF) for CYP2C9 using the N1 probe (blue: negative energy of interaction; yellow: positive energy of interaction). (b) MIF filtered by cut out option in GOLPE and considering only the negative interactions for the N1 probe (blue: negative energy of interaction). (c) Selected points by ALMOND using 1000 seeds and 50% weight for the distance-energy criteria. (d) Descriptors calculated for the different interaction fields. (e) Representation of the new descriptors in the different bin distances.

using a similarity analysis method (Figure 1), and the hydrogen atoms of the substrate are ranked according to similarity value.

Protein Treatment. Molecular interaction fields (MIF) in the binding site of the CYP 2C9 were calculated by the GRID methodology.[22] Three MIFs were generated in this analysis using the DRY (hydrophobic probe), the N1 probe (amide nitrogen hydrogen-bond donor), and the O probe (carbonyl oxygen hydrogen-bond acceptor) with a grid step size of 1 Å. The MIFs were generated using the flexible mode in GRID (directive MOVE = 1).[23] In this mode the conformationally flexible amino acid side chains of the binding site can automatically move depending on their attractive or repulsive interactions with the probe. This side chain flexibility in GRID approximately mimics the movement of the side chains as they accommodate different substrates depending on their size, shape, and interaction pattern. However, flexible grid maps do not take into account movements of the protein backbone. To define the grid box size, 50 docking solutions for the CYP 2C9 competitive inhibitor sulfaphenazole[1] were used, and the box was calculated by adding 5 Å to the maximum and minimum coordinates obtained from the ensemble of the docked structures. The ensemble of all docked solutions was used to define the possible CYP 2C9 binding pocket. GOLD[24] was used for this preliminary docking without any restriction and/or constraint and with a radius of 30 Å from the heme. The radius was set to 30 Å in order to have a wide representation of the active site. Regions close to the binding site but not accessible to the substrates were removed from the analysis by applying a preliminarily cut-out procedure to the MIFs originally computed by GRID (Figure 2a). The template needed in this cut-out procedure was built from the assembly of 50 sulfaphenazole docked structures by selecting the grid points located within 4 Å from any atom of the docked structures (Figure 2b), and these MIFs after the cut out pretreatment had 14877 interaction points (29 × 27 × 19). Since all the docked solutions were considered in the cavity definition, no restriction in the energy or score of the solution was introduced, consequently only the information about the space needed to dock the compound was extracted. However, not all the points provide relevant information. To select the most representative ones, the pretreated MIFs were exported into the ALMOND program.[25] This program selects MIF interaction points (seeds) according to an experimental design technique (D-optimal) which uses the energy of the interaction and the distance between the grid points as selection criteria

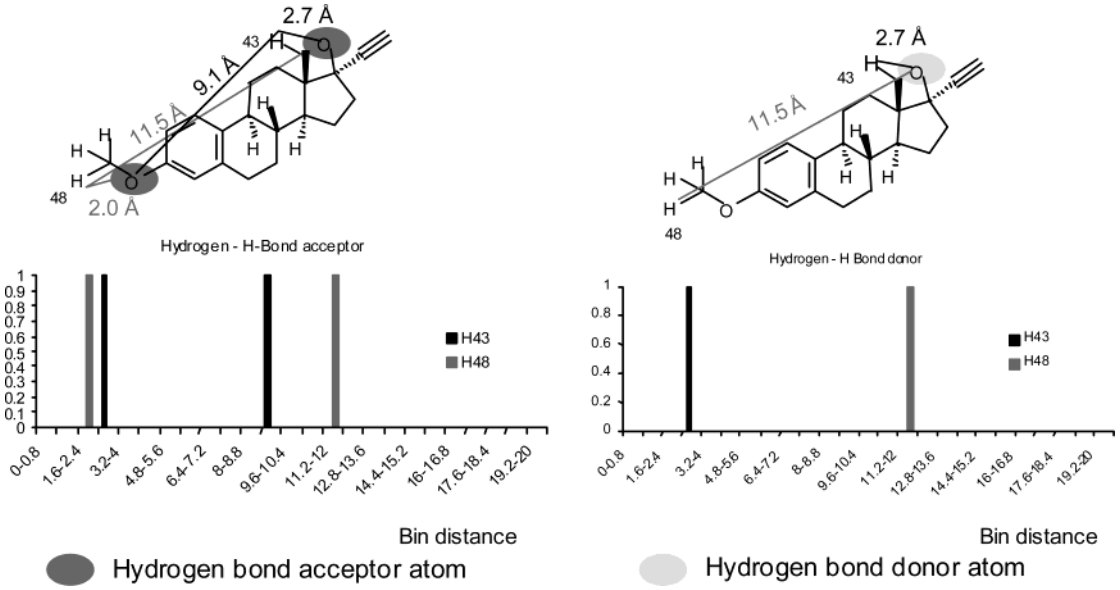


Figure 3. Ligand treatment in a steroid example. Two hydrogen atoms are highlighted: 48 and 43. The hydrogen abstraction is reported at the H48. (a) Hydrogen bond acceptor: two atoms are in this molecule that could be hydrogen bond acceptors. The distances between the hydrogen atoms (43, 48) and the hydrogen bond acceptors and the correspondence in the fingerprint are shown. (b) Hydrogen bond donor: one atom is present in this molecule that could be a hydrogen bond donor. The distances between the hydrogen atoms (43, 48) and the hydrogen bond donor and the correspondence in the fingerprint are shown. The compound conformer was calculated using CONCORD software.

(Figure 2c). In this study 50% influence of each of these factors and 1000 seeds were considered.

The selected points and the pretreated fields were then used to calculate a new set of descriptors (Figure 2d). These descriptors transform the interaction energies at a certain spatial position to a binned distance space, similar to the maximum value of the autocorrelogram in the Grid Independent descriptors procedure developed by M. Pastor et al.[25] The distance space is binned using 0.8 Å as a bin resolution; i.e., interactions between 3.2 and 4 Å will be put in the same bin. To calculate these distances, the starting point is always fixed at the oxygen atom attached to the iron atom at the reactive center of the enzyme, and the second point is then selected by the ALMOND program. For each bin distance in each field, the maximum interaction (minimum energy of interaction) corresponding to the selected point is then used in the subsequent description.

Ligand Treatment. The atoms in the CYP 2C9 substrates were classified into three GRID categories depending on their hydrophobic (DRY), hydrogen bond donor (N1), or hydrogen bond acceptor (O) capabilities. This classification of substrate atoms was done in a simplistic way using the Tripos forcefield atom types.

To deal with conformational flexibility of the substrate molecules three different types of structure were used for the analysis. The first two were obtained from a 2D to 3D conversion using CONCORD[26] and CORINA.[27] The third was obtained from a conformational analysis with a Monte Carlo search[28,29] on the CORINA generated 3D structures using the Tripos force field in Sybyl, considering a maximum of 25 conformers per compound.

The distances between the different atom positions classified using the previous criteria were then transformed into binned distances (Figure 3). In this case, the distances between the different hydrogen atoms and classified atoms were calculated, and a value of one or zero was assigned to each bin distance indicating the presence or the absence of such distance in the substrate. One set of descriptors was calculated for each category of atom types: hydrophobic, hydrogen bond acceptor, and hydrogen bond donor atoms, yielding a fingerprint for each hydrogen atom in the substrate molecule.

(c) Ligand−Protein Comparison. Once the original protein interaction patterns have been translated from coordinates into distances from the reactive center of the enzyme, and the structure of the ligand has been described as a fingerprint/hydrogen atom, both sets of descriptors can be compared (Figure 4). The Carbó similarity index was used for this purpose (eq 1, Figure 4),[30] and three similarity indexes were obtained for each hydrogen atom in a ligand:

1. The hydrophobic interaction between the protein and the ligand (hydrophobic complementarity).

2. The protein donor interaction (hydrogen bond donor descriptors for the protein and hydrogen bond acceptor descriptors for the substrate).

3. The protein acceptor interaction (hydrogen bond acceptor descriptors for the protein and donor for the ligand).

Finally, the different hydrogen atoms of the CYP 2C9 substrate descriptors were ranked according to the computed total similarity index. Other oxidative metabolic reactions that do not extract a hydrogen atom from the substrate were not considered in the analysis.

(d) Analysis of the Prediction Capabilities. To measure the prediction capability of the method two approaches were used:

1. In the first approach the described ranking position of the abstracted hydrogen atom in the substrate is related to the computed list of hydrogen atoms using the total similarity indexes. The highest ranked hydrogen atoms in the computed list would indicate the most probable predicted sites of metabolism.

2. The second approach considers the difference between the total similarity index for the hydrogen atom that is abstracted (known from the experimental data) and the maximum predicted index found for any hydrogen atom in the molecule. This difference will be zero if the prediction matches the reported site of metabolism, and the actual difference indicates how close the prediction matches the experiment. For example, if the hydrogen atom abstracted is the second one in the ranking list, but the difference in the total similarity to the first one is small, this would indicate that there was a high probability that the second hydrogen atom is abstracted.

(e) Metabolic Reactions. The 87 metabolic reactions reported in the metabolite database,[31] only reported to be catalyzed by the CYP 2C9, were used to validate the methodology. This set of reactions includes 43 different substrates: 25 show one single site of metabolism (Table 1), three present two sites of metabolism (Table 2), four present three

$$\text{Similarity}(H-CYP2C9) = \frac{\sum_{i=1}^{\text{bin distance}} E_i * I_i}{\sqrt{\sum_{i=1}^{\text{bindistance}} E_i^2} * \sqrt{\sum_{i=1}^{\text{bindistance}} I_i^2}}$$

Total similarity =
Similarity (H-CYP2C9)DRY+
Similarity (H-CYP2C9)N1+
Similarity (H-CYP2C9)O

Equation 1

E_i: Energy of interaction in the protein
I_i: Presence of the distance in the ligand

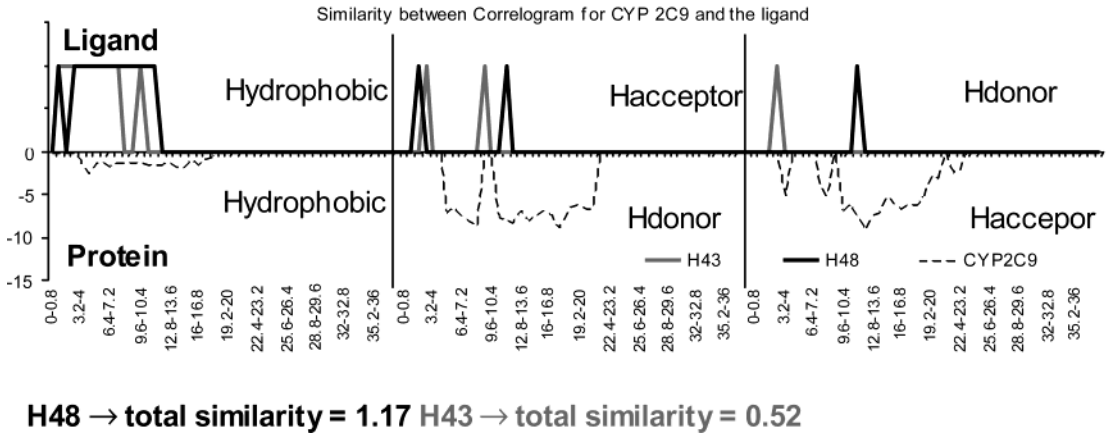


Figure 4. Comparison between the molecule fingerprint and the descriptors generated using the CYP 2C9 homology model and the grid molecular interaction field.

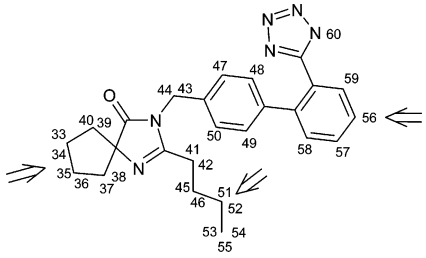


Figure 5. Substrate with multiple sites of metabolism.

sites of metabolism (Table 3), and 11 present four sites of metabolism (Table 4). Moreover, substrates show a large structural diversity including rigid compounds, (i.e., steroids) and very flexible ones with more than 10 rotatable bonds and a wide range of molecular weight and lipophilicity. Only one family of 13 coumarin-like substrates is in this data set. When the substrate has multiple sites of metabolism, the analysis uses two types of scoring to assess the ranking of all the metabolically labile positions:

The first scoring method considers each reaction independently, even if the same substrate is involved. For example, substrate **1** has three sites of metabolism (Figure 5), and if those positions appear first, second, and fifth in the predicted ranking list, those will be the numbers used as criteria to evaluate the method.

The second scoring method takes account of the fact that reactions with the same substrate should be considered at the same time when the score is computed. In the example for compound **1** this evaluation method will yield ranking 1 for the first and second hydrogen atoms and 3 for the fifth one.

Results and Discussion

The predictions for all the reactions are summarized in Figure 6. In more than 50% of cases the first option selected by the methodology agrees with the experimentally determined one. Moreover, in more than 25% and 15% of the cases, respectively, the second and third hydrogen atoms are the ones that fit the experimental findings. Moreover, in considering the second scoring method (see second point in Materials and Methods), in more than 90% of the reactions the methodology predicts the site of metabolism for CYP2C9 within the first three selected hydrogen atoms, independently of the conformer used. When considering the evaluation using the ranking list with independent reactions, more than 70% of the reactions are well predicted irrespective of the generated structure method (CORINA, CONCORD, or random conformation search).

To illustrate the outcome and validation of the methodology, some examples will now be considered classified by the type of substrate:

(a) Rigid Substrates with One Reported Site of Metabolism by CYP 2C9. The first reaction in Table 1 is used to exemplify a rigid substrate for CYP 2C9. Mestranol is the component of some oral contraceptive formulations that must be demethylated to its active metabolite, 17α-ethinylestradiol to produce estrogenic activity.[32] In this case, the hydrogen atoms selected as the most reactive ones are the same in the CONCORD and CORINA, which is the methyl group that is actually the site where the metabolic reaction is taking place. The methodology recognizes that the methoxy group compound in mestranol could be used to design a prodrug for the active metabolite: 17α-ethinylestradiol.

(b) Flexible Substrates with One Reported Site of Metabolism by CYP 2C9. The first example in this class of compounds is the O-demethylation of indomethacin (reaction 2 in Table 1). Indomethacin, a medium size compound with more than three rotatable bonds is a widely used nonsteroideal antiinflammatory drug.[33] The

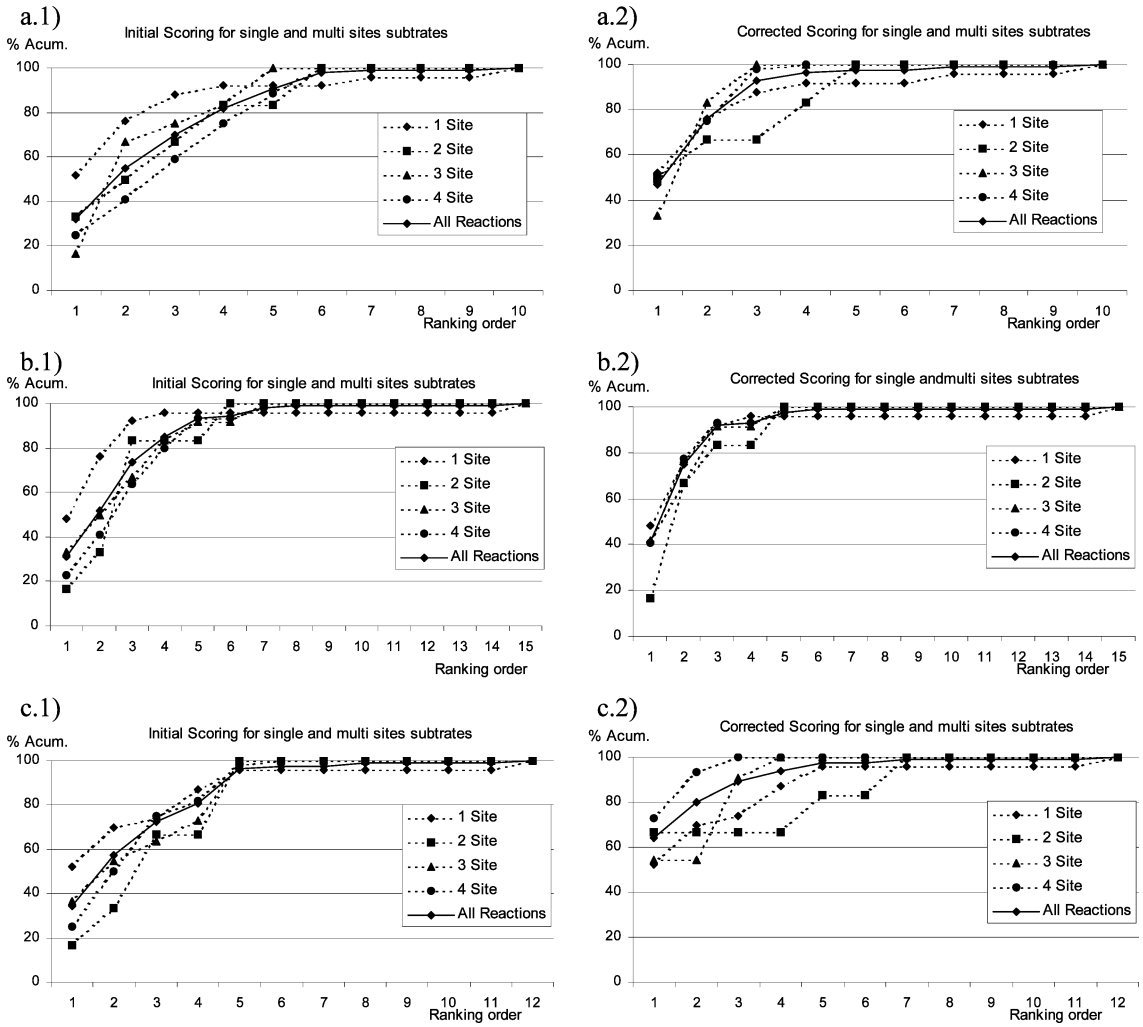


Figure 6. Hydrogen atom ranking for (a.1) CORINA-generated conformer using the 1st ranking scoring; (a.2) CORINA-generated conformer using the second ranking scoring; (b.1) CONCORD-generated conformer using the 1st ranking scoring; (b.2) CONCORD-generated conformer using the second ranking scoring; (c.1) Average for the 25 random-generated conformer using the first ranking scoring; (c.2) Average for the 25 random-generated conformer using the second ranking scoring.

O-demethylation is critical for the elimination of indomethacin and represents 40−55% of the total drug eliminated in the urine. The CYP 2C9 has been recognized as the cytochrome responsible for this degradation. The hydrogen atoms at the methyl are selected as the first one by the CONCORD-generated structure and as the second one from CORINA one and second from the average of 25 random-generated conformers. The first hydrogen atom selected in CORINA and the average of the flexible analysis is the one in the ortho position with respect to the chloro atom in the phenyl ring. Therefore, to improve the availability of this compound, the methyl group and perhaps the phenyl ring should be protected against oxidative reactions catalyzed by CYP 2C9. In fact a compound series has already been patented in the antiinflammatory research area, which protects both those indomethacin sites. [34]

A second example for a flexible molecule with one single site of metabolism catalyzed by CYP 2C9 is zafirlukast (reaction 3 in Table 1). This compound is a cysteinyl leukotriene antagonist used to treat allergic and exercise-induced asthma.[35−37] It has a rather high molecular weight (575.69) and seven rotatable bonds. The CONCORD method predicted that the observed site of metabolism would be first in the ranking list, and it was second in the list for the CORINA structure. In this case, however, the difference between the first two hydrogen atoms in the ranking list was less than 0.002. Moreover, the average results from the conformational analysis also placed the correct hydrogen atoms at the top of the ranking list. Once again the availability of this compound should be improved by protecting the predicted site of metabolism and, as before, a compound series has already been patented protecting this chemical moiety.[38]

Rigid Compounds with Multiple Sites of Metabolism. Diphenylhydantoin (phenytoin) is a drug representative of rigid compounds with multiple sites of metabolism. This drug was introduced as an antiseizure medication in 1938 by Merritt and Putman[39] and has been identified as a CYP 2C9 substrate.[33−35,40−42] The ranking list of the two hydrogen atoms involved in these reactions (Table 3) shows that both of them are at the first and second positions independently of the conformer used for the calculation.

Flexible Compounds with Multiple Sites of Metabolism. Irbesartan is an example of a flexible compound with multiple sites of oxidation (Table 3). Irbesartan is a synthetic nonpeptide antagonist of angiotensin II marketed for the treatment of hypertension, and

Table 1. Metabolic Reactions Used to Validate the Site of Metabolism for CYP 2C9 with One Reported Site of Metabolism. Table Numbering: First Row, Reactions 1 and 2; Second Row, Reactions 3 and 4, etc.

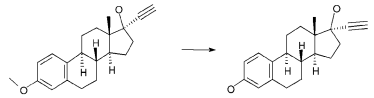
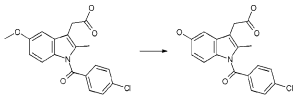
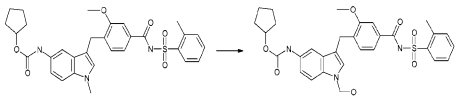
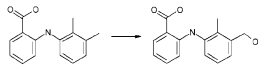
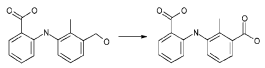
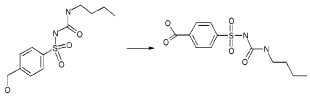
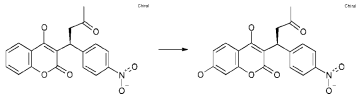
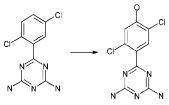
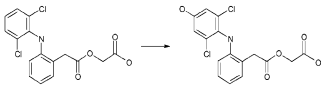
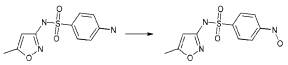
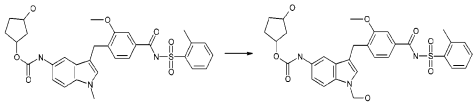
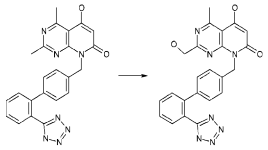
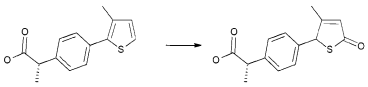
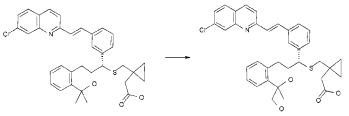
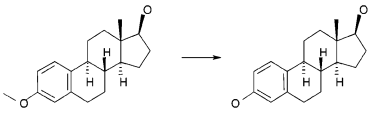
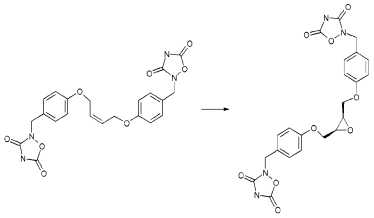
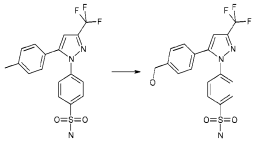
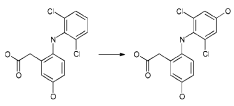
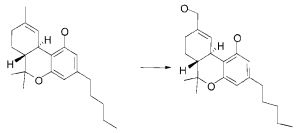
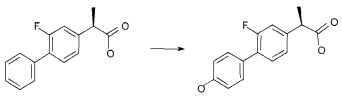
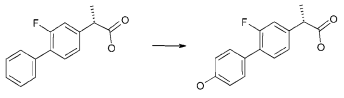
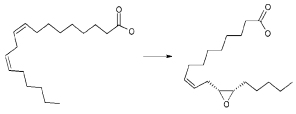
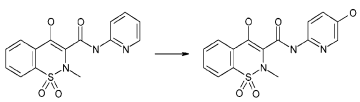
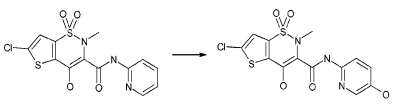
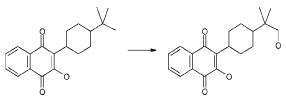
Ref	Reaction	Ref	Reaction
32		33	
35		45	
37		46	
47		48	
49		50	
35		51	
52		53	
54		55	
56		57	
58		59,60	
61		62	
63		64	
65			

Table 2. Metabolic Reactions Used to Validate the Site of Metabolism for CYP 2C9 with 2 Reported Sites of Metabolism

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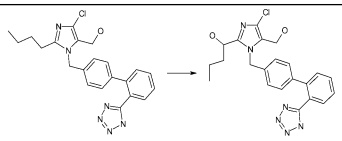
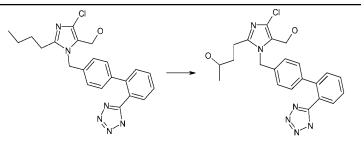
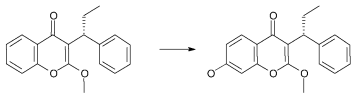
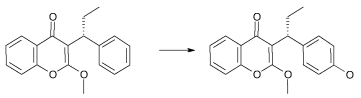
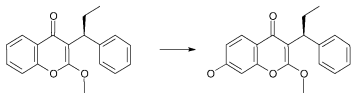
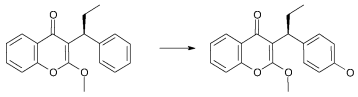
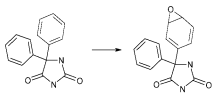
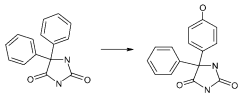
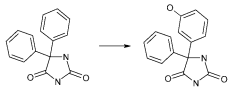
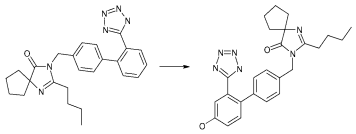
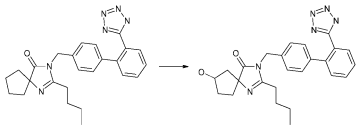
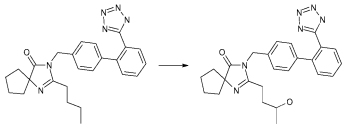
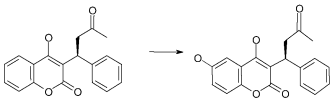
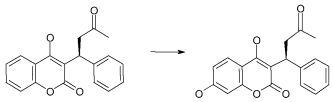
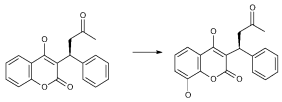
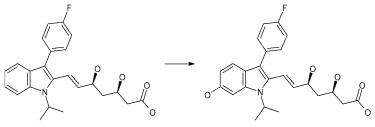
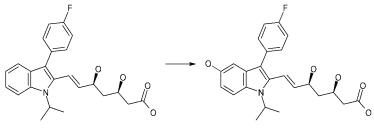
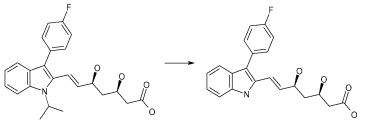
66		66	
67		67	
67		67	

Table 3. Metabolic Reactions Used to Validate the Site of Metabolism for CYP 2C9 with Three Reported Sites of Metabolism

Ref	Reaction	Ref	Reaction
40		41	
42			
43,44		43,44	
43,44			
68		68	
68			
69		69	
69			

cytochrome CYP 2C9 is primarily responsible for the oxidative degradation of this compound (Figure 5).[43,44]

In this case molecular conformation critically influences the ranking of the hydrogen atoms for metabolism because different conformations present different hydrogen atoms near the heme.

The analysis of the 25 substrate conformations involved these reactions (Table 1 and Figure 7) shows that the hydrogen atoms that are at the top of the ranking list are usually in the positions where the reactions actually occurs (Figure 5). In some cases, however, the hydrogen atom selected by the method is actually

Table 4. Metabolic Reactions Used to Validate the Site of Metabolism for CYP 2C9 with Four Reported Sites of Metabolism

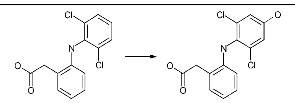
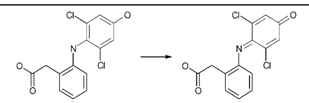
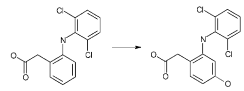
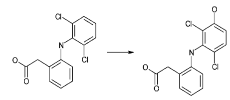
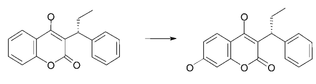
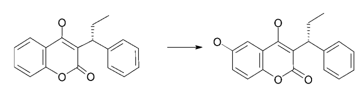
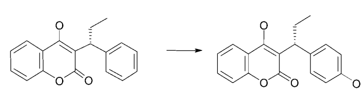
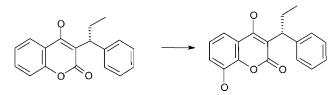
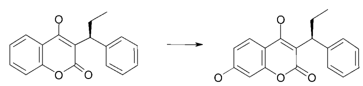
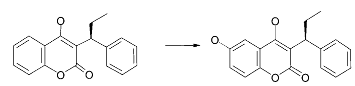
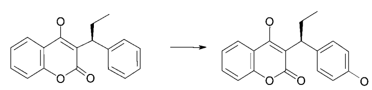
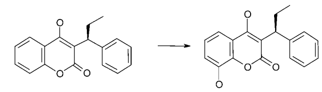
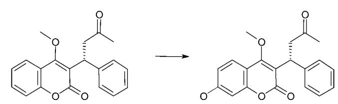
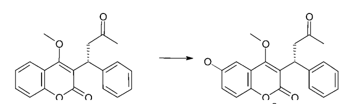
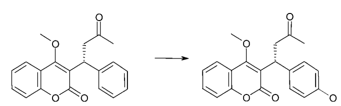
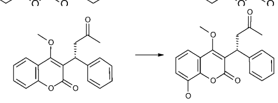
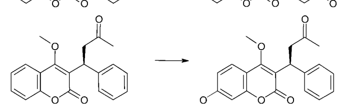
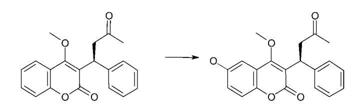
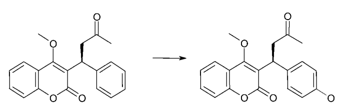
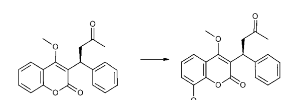
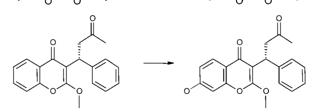
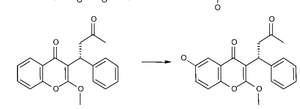
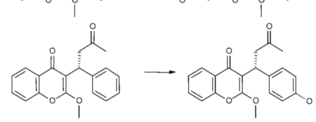
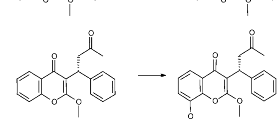
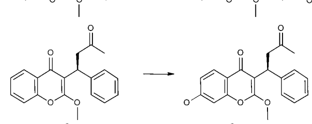
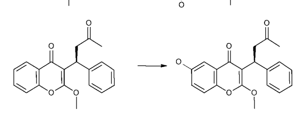
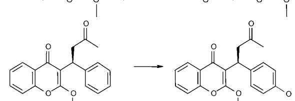
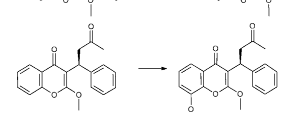
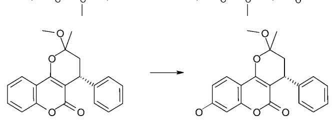
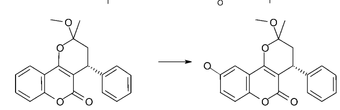
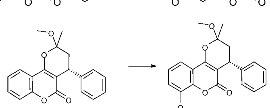
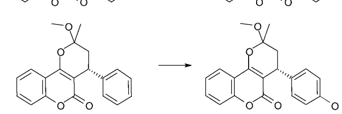
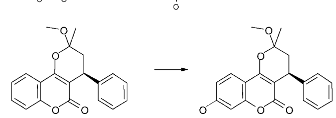
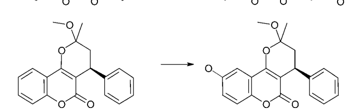
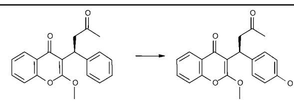
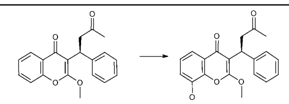
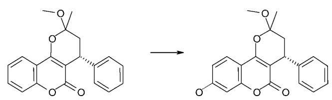
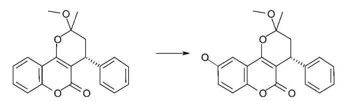
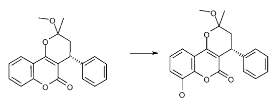
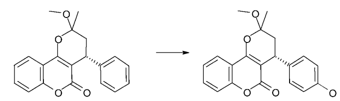
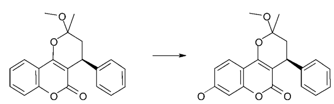
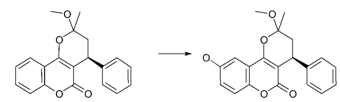
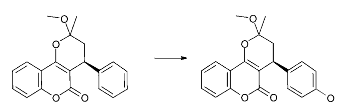
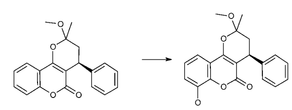
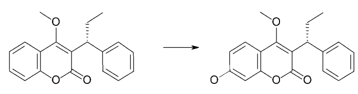
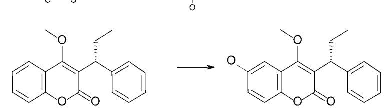
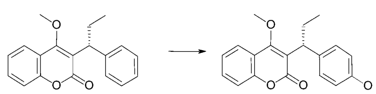
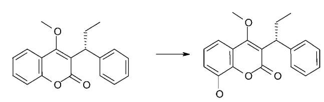
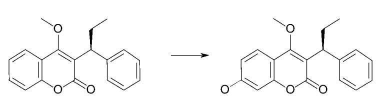
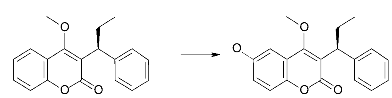
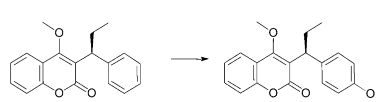
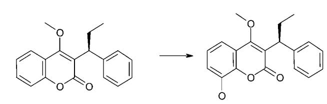
Ref	Reaction	Ref	Reaction
70		71	
59		72	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	

Table 4. (Continued)

Ref	Reaction	Ref	Reaction
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	
67		67	

attached to the carbon atom next to the observed site of oxidation. For example, experiment shows that the hydroxylation in the aliphatic side chain occurs in the methylene band (positions 51 and 52 in Figure 5), but the prediction method suggested that the most relevant hydrogen atoms were 53 and 54, which correspond to the methyl group close to the methylene. Since the method does not take into account chemical reactivity, it showed that the abstraction of a hydrogen atom from the methyl was more favorable than from the methylene, although any chemist would have known that secondary carbon radicals formed during the reactions are more stable than the primary ones and would probably have reinterpreted the prediction correctly.

As proposed in Materials and Methods, another way to analyze the data from different conformers would be either to average the ranking position obtained for each hydrogen atom in the different conformers (Figure 6c) considering all reactions independently, or alternatively by making an appropriate correction. For example, the method can indicate that hydrogen atoms 56, 35, and 51 are in the first, second, and fourth positions (Figure 6a). In the first analysis, these three positions would be considered independently, while in the second case, the ranking would consider the three sites of metabolism and would rank hydrogen atoms 56 and 34 as the first two, while hydrogen atom 51 would be the third predicted site.

Conclusions

A methodology has been developed to predict the site of metabolism of compounds, which are known to be metabolized by CYP 2C9. In more than 90% of cases, the method correctly predicted three hydrogen atoms which would most probably be metabolized.

This novel method is based on molecular interaction fields generated by GRID on a homology model of CYP 2C9. These fields were pretreated and filtered to extract the most relevant information, and the CYP 2C9 substrates were represented as a set of fingerprints for each hydrogen atom present in the molecules. This methodology can predict the site of metabolism by CYP 2C9 without the need for any time-consuming semiempirical or ab initio computations. The method does not use any training set and has been shown to be predictive for an extensive diverse validation set of ligands.

Of course the conformation of the substrate to be analyzed has an impact on the outcome of the method. Similar results were obtained with two alternative 2D-3D methods for predicting conformation (CORINA and CONCORD) and a third method which uses average of the ranking for all the conformers was also used.

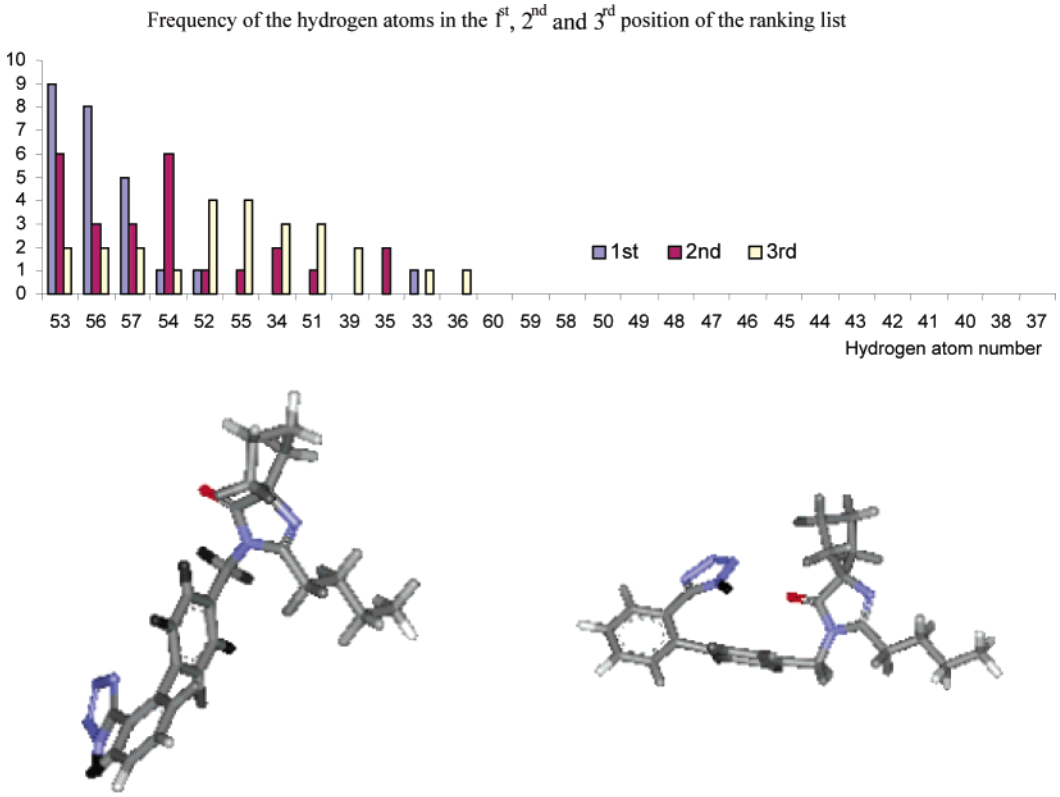


Figure 7. Hydrogen atom ranking for a flexible multisite of metabolism molecule Irbesartan: (a) Frequency of the first three ranking positions of all hydrogen atoms. (b) Two different conformations that give different rankings (the ranking order for the hydrogen atoms is shown as grey scale: white means high total similarity; black means low total similarity).

Conformation had the biggest impact in the case of flexible molecules with multiple sites of metabolism because different conformers lead to the selection of different hydrogen atoms as the first option in the ranking. In this case, the analyses of the different conformers with and without corrections for the several sites of metabolism increase the predictive power of the model.

A final but important discovery is the finding that our results can suggest new positions on ligand molecules, which should be modified in order to avoid metabolic degradation or to promote the release of an active compound from a prodrug molecule.

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JM021104I