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**RESEARCH ARTICLE**

# Pharmacokinetic parameters and a theoretical study about metabolism of BR-AEA (a salbutamol derivative) in rabbit

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**Abstract**

In this study, we report the pharmacokinetics of 1-(4-di-hydroxy-3,5-dioxa-4-borabicyclo[4.4.0]deca-7,9,11-trien-9-yl)-2-(tert-butylamino)ethanol (BR-AEA). This compound was identified as a more potent $\beta_2$ adrenoceptor ($\beta_2$AR) agonist than salbutamol. A sensitive and reproducible high-performance liquid chromatography (HPLC) method was used for determining the time-dependent BR-AEA concentration in healthy rabbit plasma. The pharmacokinetic parameters obtained are explained in relation to the compound's metabolism by sulfotransferases. For this purpose, docking simulations were carried out on SULT1A3, SULT1C1, and SULT1A1 3-D models using the Autodock 3.0.5 program. According to the HPLC results, $t_{1/2} = 2.36 \pm 0.18$ h and $K_e = 0.32 \pm 0.02\ h^{-1}$ for BR-AEA in rabbit plasma. Thus, BR-AEA has a greater half-life compared with salbutamol ($t_{1/2} = 0.66 \pm 0.08$h). This could be due to the protection that the boronic acid moiety of BR-AEA offers to the hydroxyl groups that would otherwise be susceptible to sulfation when exposed inside the active site of the sulfotransferase. This could be due to the fact that BR-AEA has a high affinity for the side-chain hydroxyl groups of Ser and Tyr residues of the enzymes, which are located outside the active site.

**Keywords:** *Salbutamol derivative; boron; $\beta_2$ adrenoceptor agonist; pharmacokinetic parameters; sulfotransferases*

## Introduction

Salbutamol continues to be the most widely used $\beta_2$ adrenergic receptor ($\beta_2$AR) agonist in the treatment of asthma.[1] There are certain diseases (e.g. asthma, chronic obstructive pulmonary disease) in which salbutamol is the first-choice drug for stimulating $\beta_2$AR.[2] However, salbutamol has some disadvantages due to its side effects,[3–5] such as tachycardia and tremor, which are caused by the fact that salbutamol crosses the lipid barrier at alveolar–capillary units after pulmonary drug administration.[6] Another disadvantage is that this $\beta_2$AR agonist has a short half-life,[7] due to its fast metabolism in the intestine, liver, and lung (in order of capacity for the metabolism of salbutamol).[8] Intestinal metabolism is triggered with oral administration but can be avoided with intravascular and inhaled administration. Thus, in the two former routes of administration, the salbutamol metabolism is considered to be almost exclusively by sulfotransferase 1A3 (SULT1A3) in lung and liver tissues[9] and in the liver also by SULT1A3 together with other SULTs, such as SULT1C1[10] and SULT1A1.[11] This is supported by the fact that sulfo-conjugates represent the major form of salbutamol-conjugates excreted in rabbits[8] and humans.[12]

Thus, it is necessary to develop and pharmacologically characterize new drugs that show affinity, selectivity, and potency in their action on $\beta_2$AR, and which also have longer duration (half-life) in the organism and fewer side effects than the agonists currently employed. Our workgroup has synthesized and identified a compound named borate of R-arylethylamine (BR-AEA), whose biological action has

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been confirmed *in vitro*.[13] The presence of a boron atom in this compound appears to be important in relation to its affinity for the $\beta_2$AR.[13] This interaction is very similar to that which occurs with other boron-containing ligands that act as inhibitors of serine proteases or other enzymes.[14]

We adapted the well-known analytical method for quantifying salbutamol[15,16] to BR-AEA, which was possible because of the similar chemical properties of the two compounds. A high-performance liquid chromatography (HPLC) technique with post-column ultraviolet (UV) detection was used for the microdetermination of BR-AEA in rabbit plasma. The results obtained from this technique show that the test compound has better pharmacokinetic parameters (a longer half-life) compared with salbutamol. Docking simulations, which were carried out on three-dimensional (3-D) models of sulfotransferases 1A3, 1C1, and 1A1 support the lower reactivity of BR-AEA for these enzymes than that of salbutamol.

## Materials and methods

### *Chemicals, reagents, and apparatus*

BR-AEA was synthesized and identified in our laboratory[13] and its structure is shown in Figure 1. Methanol and acetonitrile (HPLC grade) were purchased from TecnoLab, and HPLC water from Fremont. Salbutamol, dichloromethane, and ethanol (reagent grade) were purchased from Sigma-Aldrich.

The post-column HPLC analysis system was assembled with a manual injector (100 µL loop), using HPLC equipment from the Hewlett Packard 1100 series. For detection, a diode array detector (UV-Vis) was used at a wavelength of 278 nm. Separation was achieved with an Alltima C18 column (150 mm × 4.6 mm i.d., 5 µm particle size) acquired from Alltech. The mobile phase water–acetonitrile (1:1, v/v) passed through the column at 40°C at a flowrate of 0.6 mL/min and was filtered through 0.45 µm (nylon 47) membranes.

### *Animals*

Drug-free plasma was obtained from healthy male New Zealand white rabbits (3.75 ± 0.25 kg) from the Bioterium of Escuela Superior de Medicina-Instituto Politecnico Nacional (ESM-IPN). These rabbits were maintained under a 12 h light–dark cycle at room temperature (20 ± 5°C) and relative humidity of 50 ± 10%. All animals in this experiment were acclimated for 1 week prior to use. Food and water were available *ad libitum*. Animals utilized in the experiment were fasted overnight (~16 h) prior to and 4 h following dosing.[17] The Animal Care Committee of ESM-IPN approved the experimental protocols, and use and treatment of the animals. Animals were handled in full compliance with our institutional policies.

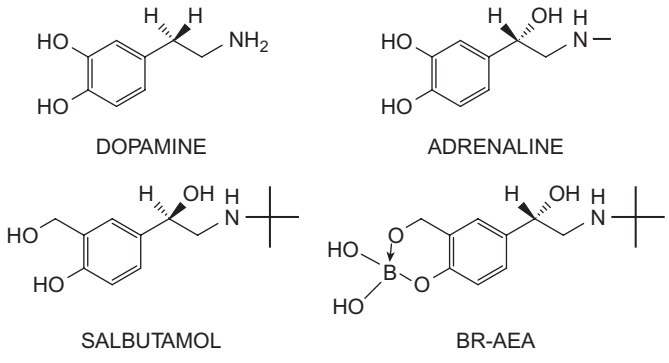


**Figure 1.** Chemical structures of ligands docked on sulfotranferases.

### *Preparation of stocks, calibration standards, and quality control samples*

The stock solution of BR-AEA (0.1 mg/mL) was also prepared in a solution composed by the same mobile phase and tested immediately. Recent calibration standards of BR-AEA in concentrations ranging from 0.5 to 10 µg were prepared in drug-free plasma.

### Sample preparations

All plasma samples, spiked plasma calibration standards, and spiked plasma quality control samples were treated in the same manner as described below.

For a plasma sample of 500 µL, 100 µL of standard solution was added to 1 mL of methanol–dichloromethane (1:1, v/v). Then it was centrifuged at 2000 rpm for 10 min at 4°C to precipitate the denatured plasma proteins; this operation was repeated three times. After that, the supernatant was passed through a Sep-Pak C18 cartridge, which had been previously washed with methanol–dichloromethane (1:1, v/v). Afterward, the sample was transferred to a conical tube and evaporated to dryness in a heated bath at 65°C; the remainder was reconstituted with 200 µL mobile phase. Finally, an aliquot (100 µL) of supernatant was placed in the HPLC system and analyzed.

### *Assay validation and quantifications*

To quantify the concentrations of BR-AEA in unknown samples, the peak-area of the compound was related to standard curves of BR-AEA in drug-free plasma.

Lower limits of detection and quantification were determined from signal-to-noise ratios.

Control rabbit plasma, obtained from three rabbits, was assessed by the procedure described above and compared with the plasma sample spiked with BR-AEA to evaluate the specificity of the method.

The absolute recovery of BR-AEA from plasma was determined for different standard concentrations (from 0.5 to 10 µg/mL) by spiking the drug into drug-free plasma. The percentage recovery was calculated by comparing the peak-areas of extracted samples with those of samples in which the compound was spiked directly in the mobile phase. Recovery of the concentration levels of three standard solutions of plasma was carried out at least three times. According to the accepted criterion, the recovery of the analyte does not need to be 100%, but should be consistent, precise, and reproducible.

### *Linearity*

For the construction of calibration curves in plasma, several calibration standards of BR-AEA were prepared and processed as described above. Calibration curves were constructed by plotting the area of BR-AEA against the known amounts of this compound. Linear regression analyses of the calibration data were performed using the equation $A = aC + b$ where $A$ is the peak-area and $C$ is the concentration of BR-AEA; unknown concentrations were computed from the linear regression equation of the peak-area and compared to the concentration of the calibration curve.

### *Precision and accuracy*

Intra-day accuracy and precision were evaluated from replicate analysis ($n = 3$) of quality control samples at different concentrations ranging from 0.5 to 10 µg/mL on the same day. The evaluation of precision was based on the criterion that the relative standard deviation should be less than ±10% for higher concentrations and less than ±20% for lower concentrations. Similarly, for accuracy, the mean value should deviate less than ±15% for higher concentrations and less than ±15% for lower concentrations.

### *Pharmacokinetics of BR-AEA*

The animals were fasted overnight but were allowed free access to water. The methodology described by Perreault *et al.*[8,18] was used for collecting samples. Thus, each of the animals received a 0.1 mg/kg dose of R-salbutamol or BR-AEA via the ear vein. This administration is commonly used in animal models, as previous processes by oral or inhaled administration affected bioavailability, showing large interindividual variations for analogous compounds.[9,18] Additionally, intravenous and endotracheal administrations have shown similar bioavailability for salbutamol.[8] Accordingly, a cannula was inserted into the contralateral ear marginal vein to sample blood, and rabbits were maintained in restriction boxes. Blood samples (3 mL) were withdrawn via the ear vein at predetermined time intervals up to 24 h after drug administration. Plasma was obtained by allowing the blood samples to stand for 1 h on ice in previously heparinized tubes, after which the samples were centrifuged at 1500 × *g* for 10 min at room temperature. Plasma samples were stored at –80°C until assayed.

### *Molecular modeling*

Dopamine, R-adrenaline, R-salbutamol, and BR-AEA were tested as ligands on SULTs. The 3-D structure of the ligands in their minimum-energy conformation was obtained by means of Gaussian 98 software using the B3LYP/6-31G* level.[19]

To identify the ligand–enzyme recognition binding sites, all the possible rotatable bonds, the torsional degrees, the atomic partial charges (Gasteiger), and merge-non-polar hydrogens of the ligands were assigned using AutoDock tool 1.5.2.[20]

The 3-D structural model for human SULT1A3 and SULT1C1 was obtained from the Protein Data Bank (PDB codes: 2A3R and 2ETG, respectively) These enzymes have high similarity to rabbit forms (Swiss-prot codes: O46640 and O46503) judging by the sequence similarity reported when using the BLAST server (≥82.3%). The 3-D structural model for SULT1A1 from rabbit is currently unavailable. Therefore, www.ncbi.nlm.nih.gov/entrez was used to search the sequence of rabbit SULT1A1 (Sequence ID: NP_001076194.1), and then BLAST was used to identify the protein sequence similarity with SULT1A2 (86.5%). In this way, a homology model of this enzyme was built based on the 3-D structure of human SULT1A2 (PDB code: 1Z28), which was generated using the SWISS-MODEL server.[21] Finally, the rabbit SULT1A1 model was structurally evaluated under Ramachandran diagrams using the Swiss-PDBViewer program.[22] Afterward, hydrogens of SULTs at pH ~7 were added and then minimized in 500 steps using the steepest descendent protocol employing the GROMOS96 43B1 parameters that are implemented in the Swiss-PDBViewer, version 3.7. The Kollman charges for all atoms of each enzyme and solvent parameters were assigned using the AutoDock tool, a program included in AutoDock 3.0.5.[20] The ligands were docked under the blind docking procedure (a rectangular grid box was built over all proteins (126 × 126 × 126 Å, with the grid points separated by 0.375 Å)) on the enzyme using the AutoDock software under the hybrid Lamarckian Genetic Algorithm, with an initial population of 100 randomly placed individuals and a maximum number of energy evaluations of $25 \times 10^7$. Resulting docked orientations within a root-mean square deviation of 0.5 Å were clustered together. The lowest energy cluster returned by AutoDock for each compound was used for further analysis. All other parameters were maintained at their default settings.[20] Ligand–enzyme complexes were visualized using the Visual Molecular Dynamics v.1.8.6 (VMD) program.[23]

## Results

### *Method development*

From rabbit plasma, BR-AEA samples were successfully separated on a Sep-Pak C18 cartridge and then on a C18 analytical column. We tested several mobile phases and found water–acetonitrile (1:1, v/v) to be the appropriate mobile phase for separation of the compounds within a run time of 2.77 min (Figure 2). We also found that the appropriate flowrate was 0.60 mL/min. If the flowrate was less than 0.6 mL/min, BR-AEA could not be separated from endogenous compounds. Typical HPLC chromatograms of BR-AEA after extraction from plasma are shown in Figure 2. No interfering peaks of endogenous compounds were found at the retention time of BR-AEA (Figure 2B and 2C).

### *Method validation*

All calibration curves were found to be linear over the calibration range of 0.5–10 mg/mL. The mean (± SD) regression equation for calibration curves in plasma was $A = 0.0373C + 0.02$; $r = 0.9992$. The recovery percentage for BR-AEA was satisfactory (Table 1). The lower limit of detection was found

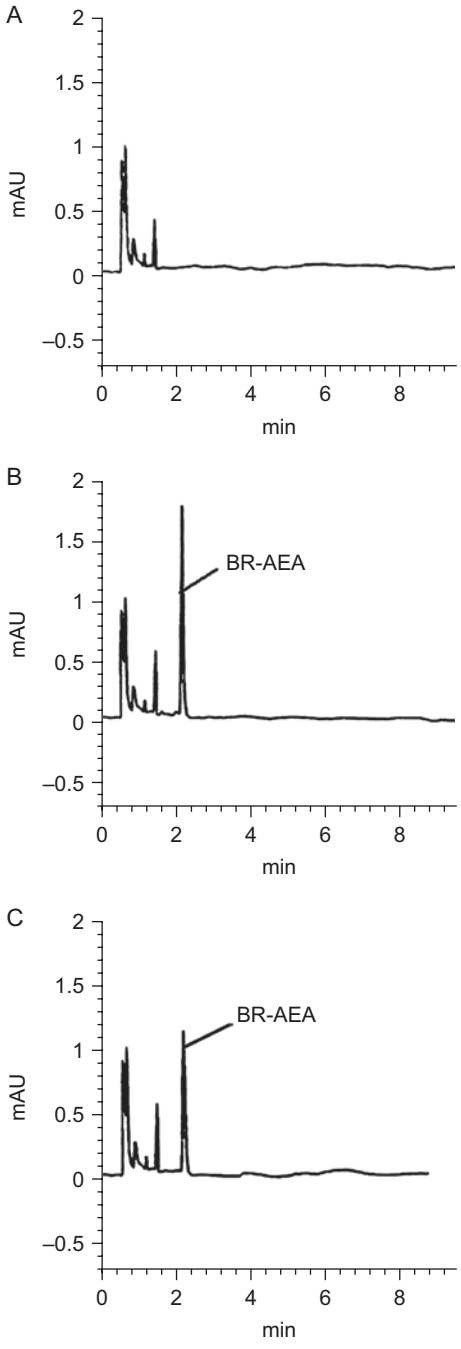


**Figure 2.** Chromatograms of (A) blank rabbit plasma, (B) plasma spiked with 1 µg/mL BR-AEA, and (C) plasma sample obtained from a rabbit at 7 h after i.v. administration of BR-AEA (0.1 mg/kg).

**Table 1.** Precision and accuracy of BR-AEA in rabbit plasma ($n=3$).

| Concentration (ng/mL) | (Obtained) intra-day concentration, mean ± SD (ng/mL) | Accuracy (% recovery) | Precision (% CV) |
|---|---|---|---|
| 500 | 457.6 ± 3.75 | 91.50 | 1.42 |
| 1000 | 954.0 ± 6.10 | 95.40 | 1.10 |
| 2500 | 2418.6 ± 28.41 | 96.74 | 2.00 |
| 5000 | 4868.0 ± 28.44 | 97.36 | 1.00 |
| 10,000 | 9620.0 ± 60.80 | 96.20 | 1.10 |

*Note*. SD, standard deviation; CV, coefficient of variation.

to be 0.40 µg/mL, whereas the lower limit of quantification in plasma from treated animals was 0.50 µg/mL.

### *Accuracy and precision*

The inter-day variability of the assays for plasma is listed in Table 1. The inter-day coefficient of variation (CV) at 0.5, 1.0, 2.5, 5.0, and 10.0 mg/mL was 1.42%, 1.10%, 2.00%, 1.00%, and 1.10%, respectively. One-way analysis of variance (ANOVA) was carried out with the grouping variable "day" (level of significance 0.05). The variation in results was not significant when data for each day were compared with those of other days and within the same day.

### *Pharmacokinetics study of BR-AEA in rabbits*

We applied the method described above for a pharmacokinetic study in which BR-AEA was administered to healthy rabbits. After a single intravenous bolus injection of BR-AEA (0.1 mg/kg), we monitored the drug concentration in plasma for 24 h (Figure 3). As can be seen for this compound and others,[24] the concentration–time data from rabbit plasma fitted a two-compartment open model. The calculated pharmacokinetic parameters of BR-AEA are listed in Table 2, which shows that BR-AEA has a greater half-life ($t_{1/2}$) than salbutamol.

### *Molecular modeling*

Docking computational methodology was used with the aim of exploring the binding site of the known ligand as well as

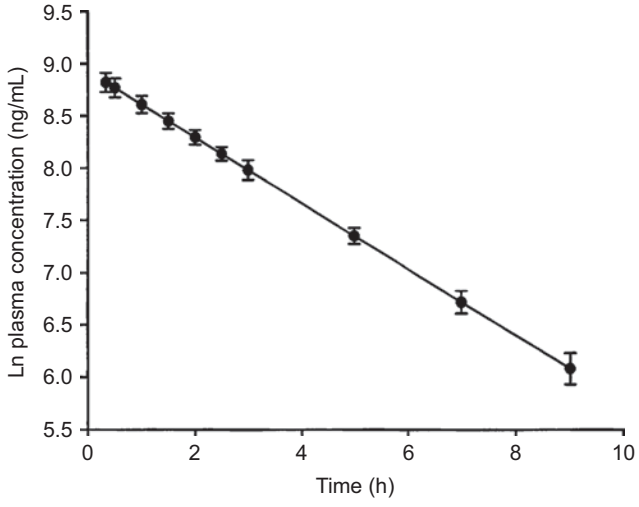


**Figure 3.** Plasma concentration–time disappearance curve of BR-AEA in rabbits following i.v. administration of 0.1 mg/kg BR-AEA. Each data point represents mean ± standard deviation ($n=6$).

**Table 2.** Pharmacokinetic parameters of BR-AEA ($n=6$) and R-salbutamol (0.1 mg/kg, i.v.).

| Compound | $K_e$ ($h^{-1}$) | $t_{1/2}$ (h) | Vd (L) | Cl (mL/min) |
|---|---|---|---|---|
| BR-AEA | 0.32 ± 0.02 | 2.36 ± 0.18 | 3.1 ± 0.02 | 95.37 ± 7.97 |
| Salbutamol[a] | 1.03 ± 0.02 | 0.66 ± 0.08 | 4.60 ± 0.50 | 80.00 ± 4.00 |

*Note*. $K_e$, elimination constant; $t_{1/2}$, half-life of the terminal phase; Vd, apparent volume of distribution; Cl, clearance.
[a]Data taken from Perreault *et al.*, 1992[18]; data included in this range were determined from our standardization process.

BR-AEA on SULT1A3, SULT1C1, and SULT1A1. The focus was to analyze specific interactions of BR-AEA with the enzymes in order to determine the pharmacological mechanism that can explain why it has a longer half-life than salbutamol. Visualizations of docked ligands on SULTs showed that all ligands at their lowest free energy conformations during the ligand–enzyme complexes interact at the binding site described for each enzyme.[10,11,25] The docking study showed that known substrates interact with the described binding site residues in each of the SULTs. The lowest free-energy conformation of these ligands was superimposed on SULTs, showing that BR-AEA has different coordinates from the other ligand structures (Figure 4). In all ligand conformational structures, the hydroxyl groups that are bonded to the boron atom of BR-AEA made hydrogen bonds at a distance of less than 3.8 Å with lateral chains of the amino acid whose exposed hydroxyl groups (Ser, Tyr, and Asp) can be either hydrogen donator or hydrogen acceptor. BR-AEA interacted with the carboxyl group at the Glu146 residue of SULT1A3 through a pair of close hydrogen bonds. The conformational binding site for BR-AEA is in a position that is not favorable for the addition of a sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Figure 5), the last amino acid demonstrated to govern the substrate selectivity of this enzyme.[24] Consequently, these structural differences of BR-AEA at the binding site of all SULTs suggest its low or null metabolism. In similar form, BR-AEA interacted by hydrogen bonds with Tyr240 on SULT1A1 and with Asp60 on SULT1C1. Furthermore, by adding an atom to salbutamol, the distances between the hydroxyl groups of the enzymes and the resulting ligand (BR-AEA) were increased, which allowed this compound to make several hydrogen bonds and block the sulfation process by the SULTs. Also, on the three SULTs, the aromatic ring of BR-AEA showed interactions with aromatic residues or non-charge moieties in its side chains (Figure 5). The hydroxyl group linked to the beta carbon and amino groups of BR-AEA make hydrogen bonds and electrostatic interactions, respectively, with some residues in the binding site of these enzymes, except in SULT1A3.The binding site of the latter enzyme has Tyr240, a residue that interacts by hydrogen bonds with BR-AEA. In its catalytic site,[25] the calculated p$K_d$ values indicated lower affinity of BR-AEA than of salbutamol for the three SULTs and lower affinity of BR-AEA than of dopamine and adrenaline for SULT1C1 and SULT1A1 (Table 3).

## Discussion

In this study, we developed a straightforward and accurate HPLC method to detect BR-AEA in rabbit plasma, which allowed us to determine its pharmacokinetic parameters, showing that it has a greater $t_{1/2}$ than that reported for salbutamol.[8,16,18]

Chemoinformatics and molecular modeling are widely used disciplines to explain the chemical and biological behavior of compounds.[15] Therefore, we applied homology modeling and docking simulations to explain some pharmacokinetic parameters of BR-AEA in the rabbit. These computational tools help show the protein in 3-D, and the affinity of small ligands with this protein. The binding mode of ligands with macromolecules on other target macromolecules is also shown.[19] The pharmacokinetic behavior of BR-AEA could be deduced as a consequence of conservation of the chemical characteristics of salbutamol that allow these compounds to avoid the action of monoamine oxidase and catechol-*O*-methyl transferase.[9–11] On the other hand, salbutamol is a compound that undergoes high liver and lung metabolism by sulfotransferases, which has been proved *in vitro*[26] and *in vivo*[8,12] for rabbits, humans, and other animal species.[16] Also, it has been proved that these enzymes could metabolize salbutamol analogs, since they are a highly selective group of enzymes that are almost exclusively responsible for the metabolism of similar substrates.[27,28]

On the other hand, the boron atom and hydroxyl groups in BR-AEA protect it from the action of SULTs, since the hydroxyl groups bound to the boron atom give it a great capacity to form stable hydroxyl bonds at a site which makes sulfation by the SULTs difficult. Therefore,

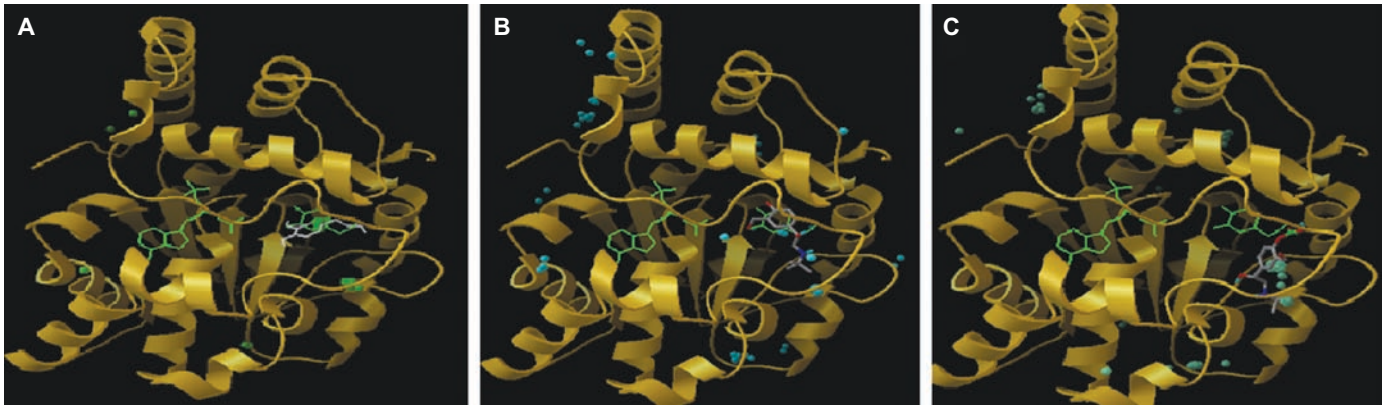


**Figure 4.** Binding sites for (A) dopamine, (B) salbutamol, and (C) BR-AEA on SULT1A3. The 100 conformations with lowest energy at interaction are represented as spheres. Dopamine and 3′-phosphoadenosine 5′-phosphate (PAPS) are depicted with the coordinates found in the crystallized structure (PDB code: 2A3R). Ligands are depicted in the lowest free energy complex. BR-AEA does not have moieties exposed for enzyme sulfatation from PAPS.

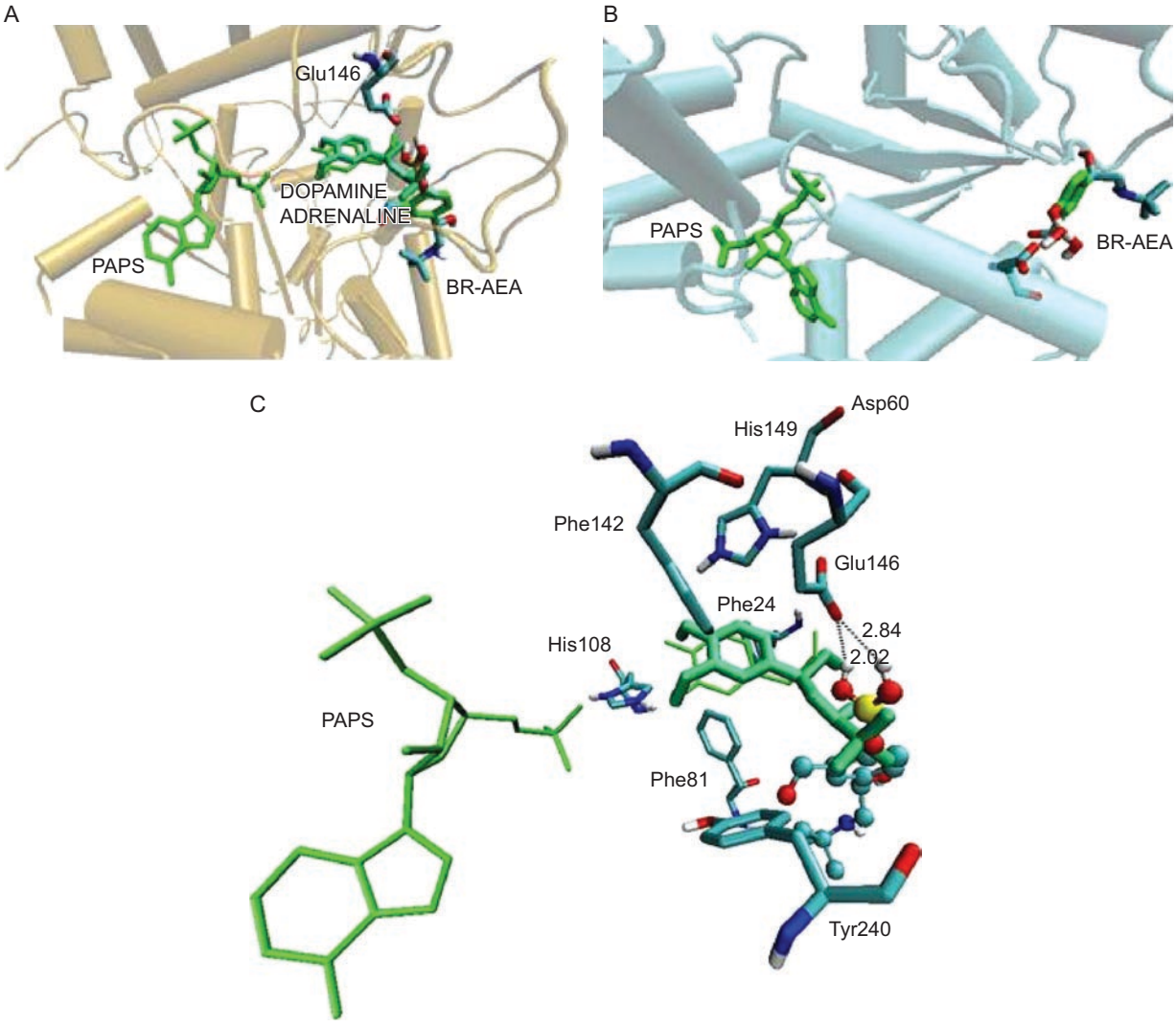


**Figure 5.** Binding of BR-AEA on SULTs. (A) BR-AEA on SULT1A3; (B) BR-AEA on SULT1C1; (C) a closer view of the binding site for salbutamol (aquamarine bonds) and BR-AEA (in stick and ball representation) on SULT1A3. PAPS and dopamine were built with coordinates from the models (PDB codes: 2A3R and 2ETG) and are shown as green bonds. Residues reported in the binding site are labeled. Interaction of hydroxyl groups bonded with Glu146 to the boron atom can be visualized.

**Table 3.** $K_d$ values of SULT substrates on SULT1A3, SULT1C1, and SULT1A1 with computational docking.

| | $K_d$ (kcal/mol (μM)) | | |
|---|---|---|---|
| Compound | SULT1A3 | SULT1C1 | SULT1A1 |
| Dopamine | –7.34 (4.16) | –10.19 (0.03) | –10.18 (0.03) |
| Adrenaline | –7.66 (2.43) | –8.58 (0.51) | –8.37 (0.73) |
| Salbutamol | –9.47 (0.11) | –7.88 (1.68) | –8.86 (0.32) |
| BR-AEA | –8.31 (0.80) | –6.78 (117) | –8.31 (0.80) |

*Note*. $K_d$, dissociation constant; SULT, sulfotransferase.

the sulfate transferring from PAPS is avoided (Figures 4 and 5). Some boronic acids have been shown to act as serine protease inhibitors.[14] Thus, the tetravalent boron atom present in BR-AEA[13] can be a potent nucleophilic center for interaction with the hydrogen of the hydroxyl groups of the amino acid lateral chains of the enzymes. Whereas in SULT1A1 and SULT1A3 the interactions are near the reported binding site,[29] this is not true for SULT1C1. For the latter enzyme, BR-AEA binds at its lowest energy in another region far from the PAPS binding site (Figure 5B). For the former two enzymes it is common to observe the same interactions with residues that have hydroxyl groups in their side chains.

On the other hand, this boronic acid moiety in BR-AEA, such as suggested by the aqueous solution UV-Vis maximum absorbance peaks at approximately 205.0 nm for this compound (different for salbutamol, 292 nm),[30] confers more hydrophilic properties to some compounds.[14,31] BR-AEA has a log P value equal to –0.410, compared to –0.316 for salbutamol.[30] Thus, this physical–chemical characteristic, which does not allow BR-AEA to cross the lipid barrier, could offer an additional pharmacological advantage.

These properties suggest that BR-AEA could provide longer-lasting action in the airways compared to some other $\beta_2$AR agonists. Hence, BR-AEA is a potentially useful drug for patients with pulmonary diseases. Additionally, the systemic side effects of this compound could be much less than

those for salbutamol, judging by the greater hydrophilicity and $\beta_2$AR-affinity[13] of BR-AEA.

## Conclusions

An HPLC method with post-column UV detection provides a new, simple, reproducible, and valid assay for the determination of BR-AEA in plasma. This method demonstrated that this compound has a greater half-life than salbutamol, apparently the consequence of the presence of a boronic acid, which, by interacting with hydroxyl groups of lateral chains of amino acids outside of the catalytic site, delays or avoids metabolism by SULTs.

More studies are necessary for evaluating the pharmacokinetic and toxicological behavior of BR-AEA. However, the pharmacokinetic properties described in this work could make it more advantageous than other $\beta_2$AR agonists in the treatment of patients with pulmonary disorders.

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