**Review Article**

**Methods used to study drug-drug interactions: A review**

**ABSTRACT**

Drug-drug interaction is a pharmacokinetic parameter which among others determines the fate of drug in giving their therapeutic actions. Most of the drug interactions are studied by using *in vitro* methods though few *in vivo* methods are also applied. The review aimed to summarise the different aspects of methods used to study the drug interactions. In order to collect relevant data on methods to study drug-drug interactions, different search engines such as Google Scholar, PubMed, Scopus and Research Gate were used for the literature survey. Alongside these search engines, standard textbooks were also consulted to extract relevant information. The findings revealed the different methods used to study drug-drug, drug-metal and drug-disease interactions. The *in vitro* methods used in drug-drug interaction (DDI) analysis included: UV-VIS absorption spectral analysis, Conductometric analysis, Ardon’s method, Job’s continuous variation method, Differential Scanning Calorimetry (DSC) thermogram, FTIR spectroscopic investigation, Thin Layer Chromatography technology, Fluorescence spectroscopy, and high-performance liquid chromatography (HPLC). Additionally, in the case of the *in vivo* method, young healthy animals having different body weights are used. Lastly, the *in silico* methods used in drug-drug interaction analysis included: Quantitative Structure–Activity Relationship (QSAR) Models, Molecular Docking and Molecular Dynamics (MD) Simulations, and the Physiologically Based Pharmacokinetic (PBPK) Modelling. Overall, the study confirmed Organic Anion Transporting Polypeptide 1B1 (OATP1B1)’s key role in the pharmacokinetics of the statins and identified reasons for DDI prediction discrepancies. In conclusion, the DDI study is an important pharmacokinetic parameter, and proper methods are needed to study this vital parameter.

**Key words**: Drug-drug interaction, Spectral analysis, conductometry, Ardon’s method, Job’s method, co-morbidity and combination therapy.

**INTRODUCTION**

The study on interaction and complexation of drug molecules with other drugs species, herbs, various metal ions and food components is an important field of research in the chemical, biochemical and medicinal point of views (1-7). Polypharmacy, that is, prescribing many drugs at a time is a common practice in case of patients undergoing a major operation, hospitalized patients, patients with co-morbidity and also in geriatric patients. Sometimes co-administration of more than two different classes of drugs may ensue effects that are neither safe nor effective but sometimes may be beneficial (8-9). Again, various mineral supplements along with drug therapy are a common and useful practice for the treatment of diseases where minerals and drugs are given concurrently. The drugs may exhibit effects independently or may interfere or interact with each other. The interaction may be agonist or antagonist of one drug by another. Sometimes, the combination therapies are beneficial to the patients and sometimes it causes serious negative effects. Patients with diseases like kidney, liver and/or heart transplantation or failure, diabetes mellitus and hypertension, anemia, bone and lipid disorders and so on are frequently prescribed numerous medications. Drug-s rug interaction might also affect the structure of the final product and cause formulation problems like tablet splitting (10). Drug-drug interaction are closely related with the drug safety and pharmacovigilance (11). Concomitant use of a large number of medications may have increased risks for drug interactions. Drug interactions are classified into two classes - pharmacokinetic and pharmacodynamic interactions. Pharmacokinetic interactions involve induction or inhibition of metabolizing enzymes in the liver or elsewhere, displacement of drug from plasma protein binding sites, alterations in gastrointestinal absorption, or competition for active renal secretion. Pharmacodynamic interactions include those that result in additive or antagonistic pharmacological effects. Therefore, drug interactions definitely alter the pharmacological effects. The effects of a moderate interaction may cause deterioration in the patient’s clinical status, resulting in additional treatment, hospitalization, and/or an extended hospital stay. The effects of a major interaction are potentially life-threatening or can lead to permanent damage of organs. Therefore, in our current review work we have summarized the different aspects of drug interactions (12–16)

**MATERIALS AND METHODS**

To collect relevant data on methods to study drug-drug interactions, different search engines such as Google Scholar, Pub Med, Scopus and Research Gate were used for the literature survey. The keywords, “DDI (Drug-drug interaction)”, “Spectral analysis”, “conductometry”, “Ardon’s method”, “Job’s method”, “co-morbidity” and “combination therapy” etc. were used to find relevant research articles and gather information on different aspects of drug-drug interactions. Alongside these search engines, standard textbooks were also consulted to extract relevant information.

**FINDINGS AND DISCUSSION**

In this section, we shall discuss different methods used to study drug-drug, drug-metal and drug-disease interactions. Basically, *in vitro* methods were highlighted.

***In vitro* methods used in drug-drug interaction analysis**

The *in vitro* interaction studies (17) are studied by observing UV-VIS absorption spectra, conductometric data, Job’s continuous variation and Ardon’s spectrophotometric methods. A description of each method is provided below. A few other studies are included (18)

**(i)** **UV-VIS absorption spectral analysis**

Spectroscopy is defined as the interaction between matter (chemical compounds) and electromagnetic radiation (simply light). Ultraviolet and visible (UV-VIS) spectroscopy is an excellent non-destructive analytical technique useful for a large number of organic compounds and some inorganic compounds. As a function of wavelength, UV-VIS spectrophotometers measure the absorption or transmission of light that passes through a solution of compounds having very low concentrations. The normal wavelength range of UV-VIS is around 200 nm to 800 nm. From 200 to 400nm is used for UV and 400-800nm is used for visible light. The measurement is based on Lambert-Beers law (19)The simple mathematical formula of this law is -

A=abC

Where,

A = Absorbance or optical density

a = Absorptivity or specific extinction co-efficient (constant).

b = Pathlength of cell (in centimeters) or thickness of the solution through which incident light passes (constant and 1centrimeter)

C = Concentration of solute (drug) in the solution(s) (gram/Liter).

This law is valid only for very dilute and transparent solutions. Absorbance is measured at different wavelengths and the obtained values are plotted by placing absorbance values at the ordinate and wavelength values at the abscissa (**Figure 1a)**.

A spectrometer is a machine that measures the optical activities of a solutions. This is performed in two ways – (i) photometric mode and (ii) Scanning mode. The response obtained from the spectrometer is called spectrum. A schematic diagram of a spectrum and a spectrometer is shown in **Figure 1b**.

|  |  |
| --- | --- |
| (a) | (b) |
| **Figure 1**: A simple schematic diagram of (a) UV-VIS spectral analysis and (b) a spectrophotometer. | |

For drug-drug and drug-metal interaction study (20), dilute solutions of individual drugs at different concentrations (for example, DRUG A and DRUG B) are prepared using different buffered pH solutions such as pH 1.4, 3.4, 6.4, 7.4 and 8.4. The absorbance of individual drug solutions is measured using definite wavelength for the drug and recorded. Then the solutions are drugs are mixed together at 1:1 ratio, stirred well for proper mixing and time is allowed for interactions to take place. Then, their absorbances are measured using specific wavelength. The absorbances and the spectrums of individual drugs are compared with the data that are obtained from the mixtures of two drugs. The experiments are repeated to avoid any error and biasness. The pH solutions are prepared using standard textbook (21)Works of other investigators can be consulted for more clarifications (22-26).

Other examples of the application of the UV-VIS spectral analysis includes in vivo protein binding studies of drugs in rat models. (27–29)

Spectrofluorometry can also be used to study drug-drug interactions (30).

**(ii)** **Conductometric analysis.**

Conductometry is a measurement of electrolytic conductivity to monitor a progress of chemical reaction one of which is drug-drug interaction, a very reversible and first order reaction. Conductometry has application in analytical chemistry, where conductometric titration is a standard technique. Conductometry is often applied to determine the total conductance of a solution or to analyze the end point of titrations that include ions. Conductometric titrations follows the general principle for acid-base titrations (31-33), Basic and its principle is based on Illkovic equation the simple mathematical form of which is –

Where,

id - In microamperes, id is the diffusion current 607 - The constant 607 is the sum of a variety of numerical factors, including the Faraday constant (P), the density of mercury, etc.

n - In electrode reactions, n is the number of electrons involved,

D - The diffusion coefficient D in centimeters per second-1,

m - Weighed by mg.sec-1 of the flow of Hg through the capillary,

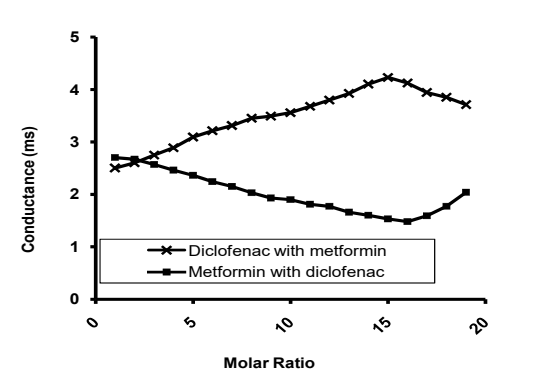
t - Time in seconds (Drop time),

C - The concentration C (mmol/L) is the concentration of electroactive solute present in the solution.

d - in the subscript form indicates the term diffusion.

In simple mathematical expression it can be expressed as –

Conductometric titrations are done to detect the complex formation of a drug (say DRUG A) with another drug (say DRUG B) as well as to find the molar ratios of the interacting agents to the drug molecule in the complex. Usually, dilute solution of one drug (40 mL of 0.005M, DRUG A) is taken in a 100 mL beaker and is titrated individually with gradual addition of another drug having a higher concentration (0.05M, DRUG B) from a burette. Reversely, 40 mL of 0.05M of DRUG B, is titrated with gradual addition of 0.005M of DRUG A. The conductance values (mS or µS) are plotted against molar ratios between the two DRUGS present in the system. The titrations curves show break at the points of possible interaction. Conductometric titrations are performed with solutions adjusted to pH 1.4, 3.4, 6.4, 7.4 and 8.4 to mimic the pH at different body chambers. The pH solutions are prepared using standard textbooks (21,34). Conversely, each of the drug having similar concentrations (40 mL of 0.005M) are titrated with standardized 0.05M NaOH solution and the conductance values (mS or µS) are plotted against volume of NaOH. A conductometric titration graph (35) is shown in Figure 2.



**Figure 2.** Conductometric titration of metformin with diclofenac at pH 7.4 [Reproduced with permission from (35)].

**(iii)** **The Ardon’s method for analysis of drug-drug interaction**

Ardon’s method is a spectroscopic method based on Lambert-Beer’s law for analysis of drug-drug interaction study. In this method, concentrations of one drug (DRUG A) are varied while keeping the concentrations of another drug (DRUG B) is fixed. The absorbance of solutions having pH 1.4, 3.4, 6.4, 7.4 and 8.4 are measured at definite wavelength specific for DRUG A and DRUG B using UV-VIS spectrophotometer. For calculations, the following Ardon’s equation is used. The Ardon equation is a spectrophotometric equation used to calculate the stability constants of 1:1 complex between drugs (36-38). The equation is -

𝐷 - is the absorbance of the mixture

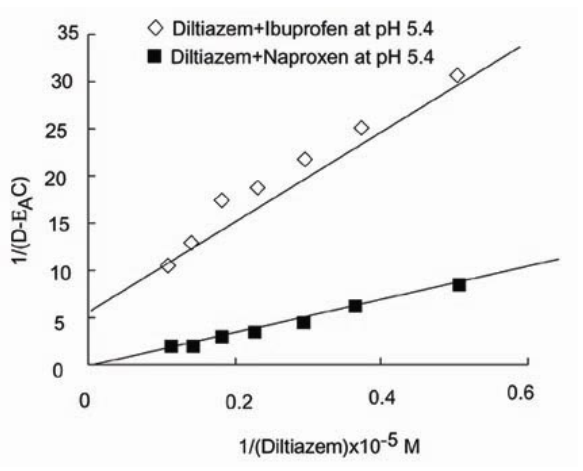
𝐵 - is the molar concentration of one drug

𝐶 - is the molar concentration of the other drug

𝜖𝑐𝑜𝑚 - is the molar extinction coefficient of the complex

𝜖𝐴 - is the molar extinction coefficient of one drug

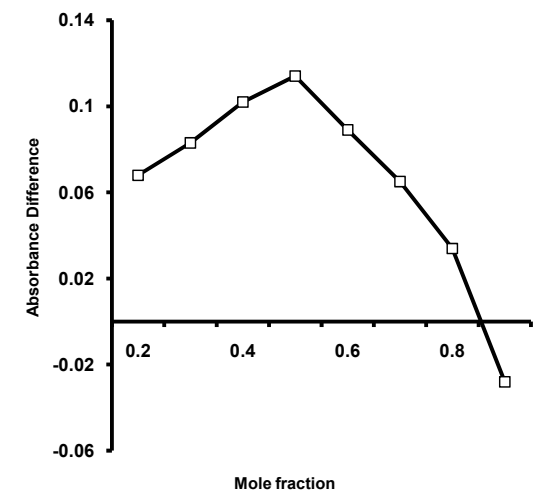
The value of n is chosen as 1, which is an essential condition for validation of the method. The values for are plotted against to get the straight or linear lines. The concentration of DRUG A is kept constant at 5x10-5M (denoted by ‘C’ in the equation). The 1:1 complex give a straight line in the plots with an intercept and slope. The stability constant of the complex is given by K=intercept/slope. It is noted that the method is only valid for the systems where 1:1 complex is formed as shown in **Figure 3**.



**Figure 3**. Ardon plot for diltiazem-ibuprofen and diltiazem-naproxen systems (26)

**(iv)** **Job’s continuous variation method**

Job’s continuous variation method is also a spectroscopic method based on Lambert-Beer’s law for analysis of drug-drug interaction study. In Job’s spectrophotometric method of continuous variation, absorbances of series of drug mixtures with various molar ratios at different pH (pH 1.4, 2.4, 6.4, 7.4 and 8.4) are measured at specific wavelength of drugs by keeping the total moles constant in the mixture. Similar experiments with reverse concentrations of drugs are performed at different pH (pH 1.4, 2.4, 6.4, 7.4 and 8.4) and the absorbances of these systems are measured at different wavelengths. The observed absorbances of the mixtures at various mole fractions are subtracted from the sum of the values for free drugs under the same conditions.



**Figure 4**. Job’s plot for metformin-diclofenac system at pH 7.4 [Reproduced from (26)]

The absorbance differences (D) are then plotted against the mole fractions of drugs in the mixtures. A curve, thus obtained, show a maximum at a point, which indicates the molar ratios of drug to drug in the complex. A Job’s plot (26)is shown in Figure 4. Similar results were also found from other studies (39,40).

**(V)** **Differential Scanning Calorimetry (DSC) thermogram**

Differential Scanning Calorimetry (DSC) is a thermos-analytical method which is used to find the difference in heat flow between an unknown sample and a standard material as a function of temperature. It gives information about thermal transitions and reactions (41).

DSC thermogram of DRUG A showed a long, sharp, characteristic endothermic peak at a particular temperature (in °C) with specific amount of Free energy (ΔH in J/g) corresponding to the melting point of DRUG A. DRUG B shows a broad endothermic peak at another temperature (°C) representing the evaporation of water molecule from the crystal lattice and an exothermic event was observed at different temperature (°C) which could be related to a crystalline state transition. DSC curves of the physical mixture have evidenced significant shifts of the endotherm compared to the endotherm of the pure drugs(42,43).

**(vi)** **FTIR spectroscopic investigation**

FTIR (Fourier Transform Infrared) spectroscopic investigation is an analytical technique that uses infrared light to analyze the chemical structure and composition of materials by examining their unique vibrational and rotational spectra(44). The FTIR imaging determines the functional groups in the studied drugs (DRUG A and DRUG B) and in their 1:1 and 1:2 mixtures. The absorption frequency corresponding to important functional groups of DRUG A and DRUG B can be obtained. The FTIR spectra of DRUG A-DRUG B complexes were judged against those of the pure drugs(45).

**(vii) Thin layer Chromatography**

Thin Layer Chromatography technology (TLC) is useful to separate a complex that has been formed as a result of interaction between two species. TLC typically employs a plate covered with silica gel, which serves as the stationary phase. The plate is “spotted” with sample solution, then placed in a jar with the appropriate solvent. Because of capillary action the solvent rises along the plate and elutes the species at different rates depending on the individual polarity. Multiple solvents are employed in order to get higher resolution of separation. The separation process is determined by the components’ respective affinity for the stationary and mobile phases. Compounds travel along the surface of the stationary phase when the TLC plate is run in solvent. Compounds with higher affinity for the stationary phase move more slowly through the plate. Furthermore, molecules with a higher affinity for the mobile phase move faster than others. Distinct spots were visualized and identified with the help of a hand-held UV light (long and short wave) as well as with the use of iodine chamber. The Rf values are calculated and used to confirm the identity of the chemical species (27).

**(viii)** **Fluorescence spectroscopy**

By using variations in fluorescence intensity, wavelength shifts, or quenching effects, fluorescence spectroscopy can identify and analyze chemical interactions. It works on the basis of the idea that certain molecules produce light when excited by a particular wavelength. Drug–drug interactions (DDIs), particularly those involving protein binding or competition for biological macromolecules, are ideally studied using this approach. Fluorescence spectroscopy is frequently employed in DDI research to track the effects of one drug on another's ability to bind to proteins, such as serum albumins. Fluorescence spectroscopy was employed to examine the interaction between tannin and bovine serum albumin (BSA), identifying hydrophobic interactions and a stationary state elimination process. Such studies highlight fluorescence spectroscopy’s utility in revealing molecular-level changes and potential pharmacokinetic consequences of DDIs. In another study, Fluorescence spectroscopy was used to study the binding of gefitinib (GEF) to human serum albumin (HSA), revealing a static quenching mechanism and moderate binding affinity (Ka = 1.70 × 10⁴ M⁻¹). The decrease in Stern–Volmer constants with temperature confirmed static quenching, while thermodynamic data suggested hydrophobic and hydrogen bonding interactions. This example shows how fluorescence spectroscopy helps identify binding strength, quenching type, and interaction sites—key aspects in drug–drug interaction analysis (46).

**(ix) HPLC**

Drugs and their metabolites can be precisely separated, identified, and quantified using high-performance liquid chromatography (HPLC), an analytical method based on the differential partitioning of substances between a mobile phase and a stationary phase. When two or more medications are supplied together, it is often used in DDI studies to track changes in drug concentrations. High sensitivity and selectivity can be attained by HPLC, depending on the detection system (e.g., UV, fluorescence, or mass spectrometry). For example, HPLC techniques have been extensively used to evaluate the effects of different substances on the plasma levels of medications that are provided together, exposing quantifiable pharmacokinetic changes. These studies demonstrate how effective HPLC is in quantitatively evaluating DDIs in preclinical and clinical contexts. In a study, An HPLC method with UV detection was developed to study the drug–drug interaction between sildenafil (SDF) and tramadol (TMD) in rabbit plasma following oral co-administration. Using a C18 column and a phosphate buffer–acetonitrile mobile phase, the method enabled simultaneous quantification of both drugs with high sensitivity (LOD: 0.05 μg/mL for TMD and 0.02 μg/mL for SDF). Pharmacokinetic analysis showed that TMD altered SDF’s Cmax, Tmax, AUC, and elimination rate, indicating a significant interaction. This study demonstrates HPLC’s strength in quantifying co-administered drugs and evaluating in vivo pharmacokinetic changes in DDI research (47). Another RP-HPLC Method for the Quantification of Omeprazole in Pharmaceutical Dosage Form was developed and validated for drug - drug interaction analysis (48).

***In vivo* methods**

In the *in vivo* studies (Md. S. Amran et al. 1970) young healthy animals (mice, rats, guinea pigs, rabbits etc.) having different body weights are used. The animals are anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Respiration is maintained by artificial ventilation through the cannula in the trachea to maintain pCO2, pO2, and pH within the normal range. A polyethylene tube is inserted into the left femoral vein to administer drugs. The common carotid artery is cannulated and connected to a mercury manometer to monitor the blood pressure. After 15 min of stabilization, saline (as vehicle or control, 0.5 ml) is injected and normal blood pressure is recorded. DRUG A (1 mg/kg body weight), and 1:1 mixtures of DRUG A + DRUG B, and DRUG A + DRUG C are administered to the different groups for each dose of the vehicle or drugs through the femoral vein as i.v. bolus injection and the subsequent effect on the blood pressure are recorded. Each animal received only one dose (treatment) of either vehicle or any of the drugs (49,50) .

**Intermolecular forces involved in drug interactions**

The intermolecular forces involved in drug interactions are - (i) van der Waals forces (dispersion, dipolar and induced dipolar), (ii) Hydrogen bonding, (iii) coordinate covalence, (iv) Charge transfer, (v) Hydrophobic interactions (1). A detailed description is out of context of this article. Interested readers may consult (1,2) for further studies.

***In silico* methods used in drug-drug interaction analysis**

1. **Quantitative Structure–Activity Relationship (QSAR) Models:**

QSAR models are computational techniques that predict the biological activity of molecules based on their chemical structure. In QSAR modeling, various chemical descriptors (such as molecular weight, lipophilicity, electronic properties, hydrogen bond donors/acceptors) are mathematically correlated with biological outcomes like enzyme inhibition or induction. When applied to DDIs, QSAR models can predict whether a new drug is likely to inhibit or induce a metabolizing enzyme (e.g., CYP450 enzymes) and thus interact with other drugs. Modern QSAR models, like those built with PASS (Prediction of Activity Spectra for Substances) or PoSMNA descriptors, offer high-throughput prediction of interactions without the need for physical testing.

In a study (51) the researchers developed computer models to predict drug–drug interactions (DDIs) mediated by seven major cytochrome P450 enzymes (CYP1A2, CYP2B6, CYP2C19, CYP2C8, CYP2C9, CYP2D6, and CYP3A4). They used about 2500 known DDI records to build structure–activity relationship (SAR) models, applying the Prediction of Activity Spectra for Substances (PASS) software and Pairs of Substances Multilevel Neighborhoods of Atoms (PoSMNA) descriptors based on molecular structures. The models achieved an average prediction accuracy of around 92%, evaluated through leave-one-out cross-validation. These SAR models are publicly available and can be used to predict potential DDIs for both existing drugs and new, not-yet-synthesized compounds.

1. **Molecular Docking and Molecular Dynamics (MD) Simulations:**

**Molecular Docking** simulates the binding orientation and affinity between a small molecule (drug) and a target protein (such as an enzyme or transporter). **Molecular Dynamics (MD)** simulations model the physical movement of atoms and molecules over time under defined conditions (e.g., temperature, pressure). Docking helps predict how strongly a drug can bind to an enzyme (e.g., CYP450, P-glycoprotein), which informs whether it might inhibit the enzyme and cause DDIs. It calculates binding energy and identifies interactions like hydrogen bonds, hydrophobic contacts, etc. Molecular dynamics refines this prediction by simulating the real-time behavior of the drug-protein complex, checking if the binding is stable, flexible, or transient. MD can uncover important phenomena such as conformational changes or induced fit effects that simple docking may miss. A study used molecular docking to investigate its interaction of GNF-351, which is a drug candidate that antagonizes the aryl hydrocarbon receptor, with ketoconazole. Researchers modeled the binding of GNF-351 to the human cytochrome P450 (CYP3A4) structure, generating 30 possible orientations. Docking results showed GNF-351 interacts with CYP3A4 through its aromatic rings, particularly near Phe302 and Phe304, similar to ketoconazole's binding position relative to the heme group. These findings suggest potential interactions between GNF-351 and ketoconazole, highlighting the need for careful monitoring if used together (52).

1. **Physiologically Based Pharmacokinetic (PBPK) Modeling:**

Physiologically Based Pharmacokinetic **(**PBPK) models are computational models that simulate the ADME (Absorption, Distribution, Metabolism, and Excretion) processes of drugs within a virtual organism based on physiological parameters.

PBPK models use real biological data (e.g., organ sizes, blood flow rates, enzyme expression levels) and drug-specific properties (e.g., solubility, permeability, metabolism rate) to create a "virtual human" or "virtual animal. These models predict how a drug moves through different tissues and how it is metabolized. For DDIs, PBPK models are used to simulate scenarios where one drug inhibits or induces the metabolism of another drug. Regulatory agencies (like the FDA) accept PBPK simulations as evidence for DDI risk assessment in drug development. Predicting drug–drug interactions (DDIs) with statins is challenging due to the involvement of both metabolizing enzymes and transporters. In a study (53), physiologically based pharmacokinetic (PBPK) models for pitavastatin and atorvastatin were developed using in vitro and clinical data in SimCYP®, accounting for both enzyme and transporter contributions. The models successfully predicted DDIs involving OATP1B1 polymorphism and interactions with itraconazole, erythromycin, and gemfibrozil but under-predicted DDIs with rifampin and cyclosporine. Further analysis suggested that existing models underestimate OATP1B1 inhibition by rifampin and cyclosporine. Overall, the study confirmed OATP1B1’s key role in the pharmacokinetics of these statins and identified reasons for DDI prediction discrepancies.

**CONCLUSION**

This review emphasised that DDI study is an important pharmacokinetic parameter and proper methods are needed to study this vital parameter. It may be underlined that only two drugs are able to induce the development of a DDI even if this clinical relevance is related to the pharmacology of each drug (Palleria et al., 2013). In fact, a DDI will be able to induce a clinically relevant effect in the presence of drugs with a low therapeutic index, a long half-life and a higher bound with plasma proteins. Mechanism-based pharmacokinetic modelling and simulation are useful for comparing hypothesised combination regimens and have multi-scale, translational potential (Niu et al., 2019).

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