



Importance and review of drug metabolite synthesis

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Cite this article as: Sahin, Z., Omurtag Ozgen, P.S., & Rollas, S. (2023). Importance and review of drug metabolite synthesis. *Istanbul Journal of Pharmacy*, 53(2), 251-269. DOI: 10.26650/IstanbulJPharm.2023.1033045

ABSTRACT

Phase I and Phase II metabolic reactions are involved in the pharmacokinetic properties of drugs after administration. These reactions mainly aim to make drugs more polar and eliminate them safely. However, some of these metabolites have the potential to exhibit a toxicological effect. Industry and/or academia have to consider these metabolites in terms of their pharmacodynamic and pharmacokinetic properties. These metabolites are not only residual intermediates from the synthetic process of the main drug but also unique structures produced by metabolic enzymes in the human organism. Thus, metabolite synthesis by synthetic or semi-synthetic methods is a key feature in the pharmaceutical industry. In this review, synthetic methods of the metabolites from all known metabolic pathways are reviewed from the literature. It was observed that both synthetic and semi-synthetic methods require more attention as they are as important and complex as drug synthesis. Moreover, it showed that there was much more research available for Phase I than Phase II in the literature.

Keywords: Drug metabolite, metabolism, synthesis, oxidation, Phase I, Phase II

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Submitted: 18.01.2022
Revision Requested: 13.05.2022
Last Revision Received: 18.05.2022
Accepted: 26.05.2022
Published Online: 28.08.2023

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INTRODUCTION

Biotransformation reactions are divided into two main classes known as Phase I and Phase II. Phase I reactions are the functionalization of a parent compound by introducing polar chemical moieties, then making them more soluble in water media. Insertion of new polar functional groups into the parent compound is performed by oxidation, reduction, and hydrolytic reactions. Phase I reactions can be carried out by either enzymatic cytochrome P450 (CYP), Flavin-containing monooxygenases (FMO), esterase, and amidases or hydrolytic at the physiological pH. In the Phase II reactions, Phase I metabolites or endogenous polar molecules are conjugated with the large hydrophilic groups (Foti & Dalvie, 2016; Low & Castagnoli, 1991). These conjugative reactions are mediated by specific enzymes (glucuronosyltransferase, sulfotransferase, and *N*-acetyltransferase) that each lead to their specific conjugate such as sulfate, glucuronate, glycine, etc. (Mulder & Burchell, 1990). Drug metabolism is one of the most important steps in ADMET studies. Thus, the synthesis of metabolites is an important process in drug metabolite profiling, metabolite stability, as well as pharmacological activity testing, metabolite quantification, toxicity testing and metabolism-based drug interaction (Rollas, 2007). The US Food and Drug Administration (FDA) guidelines for metabolites in safety testing declare the acceptable metabolite/drug ratios in drug development (Food Drug Administration (FDA), 2008). Metabolites above the 10% parent drug should be subjected to safety testing in terms of tolerability according to the metabolites in safety testing (MIST) approach by a group of scientists (Baillie et al., 2002). The approach that finds the abundance is more important than the percentage causes a debate. In this discussion, scientists from the pharmaceutical industry proposed that the abundance approach should be considered as it is more reliable in terms of dose, chemical structure, and various parameters (Smith & Obach, 2005). The development of synthesis methods is needed to obtain authentic metabolites. Particularly, the synthesis of metabolites has a crucial role in pharmaceutical industry operations for producing a large number of pure metabolites

to perform pharmacokinetic and pharmacodynamics studies. Biotransformation reactions, from the leading pharmacological activation of drugs, have been involved in aliphatic or aromatic carbon hydroxylation, epoxidation, heteroaromatic oxidation, reduction, glucuronidation, sulfation, acetylation, and other metabolic pathways. The synthesis of metabolites that are not easily carried out by chemical methods can be produced by microbial biotransformation (Asha & Vidyavathi, 2009; de Paula et al., 2015; Di Nardo & Gilardi, 2012), using the plant cultured cells as a biocatalyst (Ishihara, Hamada, Hirata, & Nakajima, 2003). Liver microsomes of various species have been used for *in vitro* metabolism studies and they are commercially available (Krebsfaenger, 2007). The biosynthesis of drug glucuronides may be performed using human liver microsomes in combination with uridine 5'-diphosphoglucuronic acid (Uldam, Juhl, Pedersen, & Dalgaard, 2011).

In this respect, this review reported several examples of the chemical and biotechnological (Schroer et al., 2010) synthetic methods of drug metabolites in favor of metabolism and pharmacologic activity studies of the pharmaceutical industry (Fox & Gibas, 1953; Fura et al., 2004; Genovino, Sames, Hamann, & Toure, 2016; Kuo et al., 2004; Lombardino, 1981; Obach, 2013) and the analysis method of drugs and their metabolites (Kostainen, Kotiaho, Kuuranne, & Auriola, 2003; Protti et al., 2020).

The synthesis of Phase I metabolites

The purpose of Phase I reactions is to introduce a polar functional group –OH, –COOH, –NH₂, –SH into the drugs and other xenobiotic molecules. These functional groups can also be released by the hydrolysis of esters or amides and the dealkylation of ethers, thioethers, and secondary amines.

Oxidation

One of the most important reactions of Phase I metabolites is oxidation. Most of the drugs, xenobiotics, and dietary compounds are metabolized by CYP enzymes which are also known as the microsomal mixed-function oxidase system. CYP enzymes are located primarily in the endoplasmic reticulum.

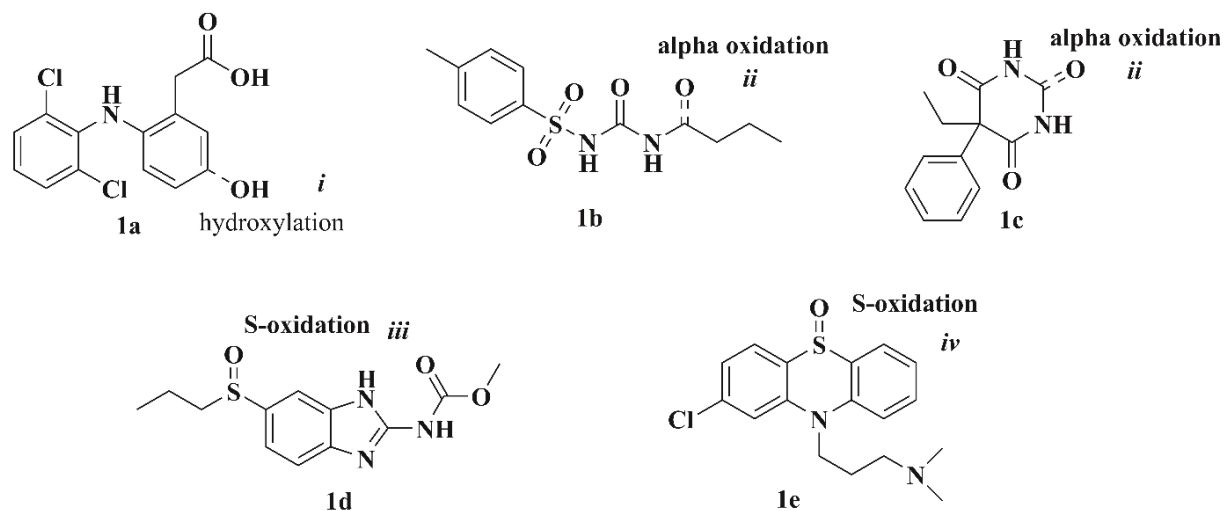


Figure 1. Structure of diclofenac (1a), tolbutamide (1b), primidone (1c), albendazole (1d), and chlorpromazine (1e) and their synthesized metabolites. (i: 10 F/mol, 4eq NaHSO₃, ACN 1:1 H₂O; ii: 6 F/mol, NaHCO₃, ACN 1:1 H₂O; iii: 1.5 F/mol, NaHCO₃, ACN 3:1 H₂O; iv: 4 F/mol, NaHCO₃, ACN).

The largest amount of CYP enzymes is found in the liver and they can also be found in intestinal, adrenal, and other tissues (Schroer et al., 2010).

Flavin monooxygenases (FMOs) are involved in the Phase I drug metabolism of a nucleophilic hetero atom containing drugs, xenobiotics, and dietary compounds to their sulfoxide or N-oxide metabolites (Gao & Zheng, 2019; Geier et al., 2015). Phase I metabolites of some drugs such as diclofenac, tolbutamide, primidone, albendazole, and chlorpromazine (Figure 1) were synthesized via preparative scale continuous-flow electrosynthesis method (Stalder & Roth, 2013).

The oxidation of aromatic and aliphatic compounds

Aromatic hydroxylation is a major route of metabolism for many drugs. Generally, hydroxylation occurs at the 4- position of the aromatic ring. Most of the phenolic metabolites undergo further conversion to polar glucuronide or sulfate conjugates.

Patrick et al. synthesized hydroxylated metabolites of methylphenidate (Patrick, Kilts, & Breese, 1981). As shown in Figure 2, the nitrile group of α -(2-pyridyl)- α -(4-methoxyphenyl)acetonitrile (**2a**) is partially hydrolyzed to the corresponding amide (**2b**) at room temperature. The direct hydrolysis reaction is not preferred because of the possibility of decarboxylation. Then **2b** is reduced by Adam's catalyst to obtain a 20:80 *erythro* / *threo* mixture of **2c**. At this stage, column chromatography or fractional crystallization manages only to isolate *erythro* configuration of **2c**. However, it is possible to separate *erythro* / *threo* mixture by fractional crystallization after hydrolysis using HBr, although the quantification for *threo* (**2d**) is quite low. Thus, first epimerization is made by using KOH to obtain *threo* compound (**2d**). **2d** and **2e** were subjected to Fischer esterification in methanol.

Acetaminophen is widely used drug as analgesic and antipyretic. Valero et al. (Valero, Lozano, Varon, & Garcia-Carmona, 2003) reported the enzymatic synthesis of catechol metabolite of acetaminophen that is not commercially available (Figure 3).

Toxic metabolites such as *N*-acetyl-*p*-benzoquinone imine (NAPQI) of acetaminophen synthesised by Dahlin and Nelson (Dahlin & Nelson, 1982) from acetaminophen and silver oxide (Figure 4).

Recombinant human CYPs expressed in *Escherichia coli* are suitable biocatalyst for the synthesis of drug metabolites. Vail et al. (Vail, Homann, Hanna, & Zaks, 2005) stated that the synthesis of anabolic testosterone metabolite 6 β -hydroxytestosterone human cytochrome P450 3A4 with NADPH-P450 reductase (NPR) was expressed in *E. coli*.

The expected human drug metabolites have been used in several model systems. One of these is microbial transformation (Asha & Vidyavathi, 2009).

Moody et al. (Moody, Freeman, Fu, & Cerniglia, 2002) produced the metabolites of antidepressant mirtazapine using the fungus *Cunninghamella elegans* as a model of mammalian metabolism. As shown in Figure 5, 8-hydroxymirtazapine obtained as a major metabolite after 96 h.

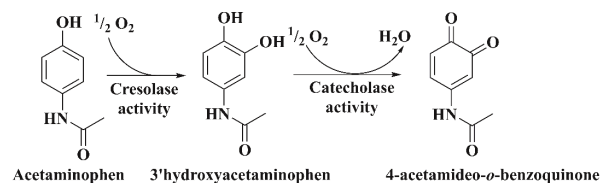


Figure 3. Representation of enzymatic synthesis of 3'-hydroxyacetaminophen from acetaminophen.

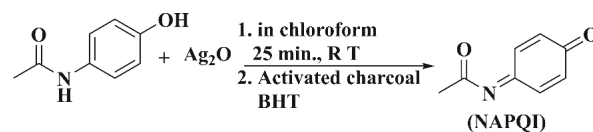


Figure 4. Synthesis of *N*-acetyl-*p*-benzoquinone imine (NAPQI) from acetaminophen.

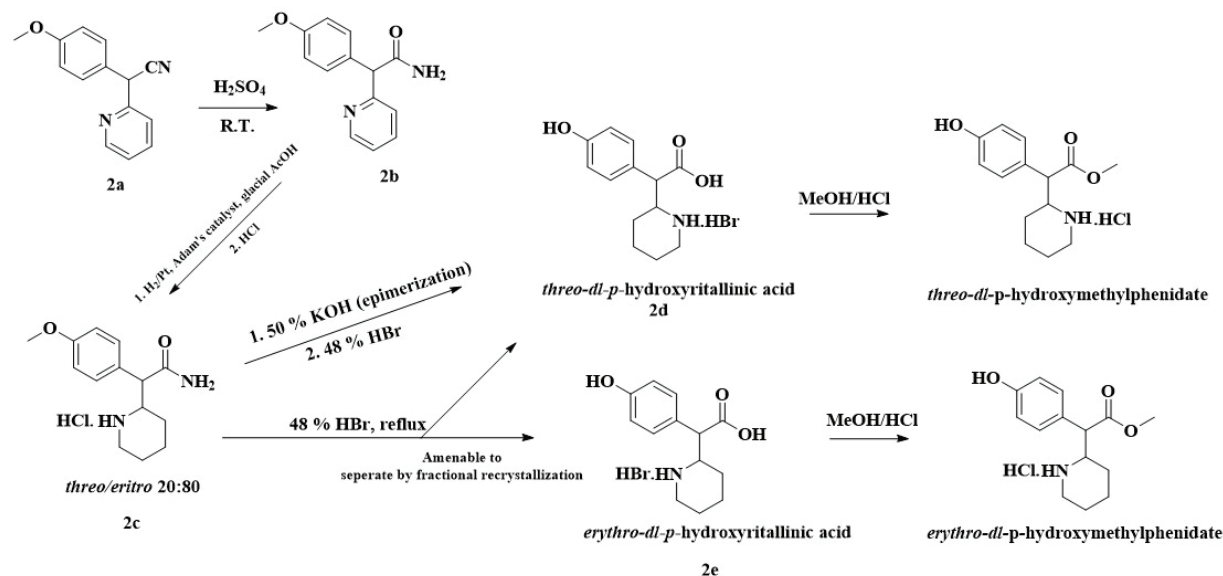


Figure 2. Synthesis pathway for hydroxylated metabolites of methylphenidate.

As shown in Figure 6, Otey et al. (Otey, Bandara, Lalonde, Takahashi, & Arnold, 2006) reported the hydroxylation of propranolol by variant P450 BM3 heme domain (BM3-H) 9C1. The variants of P450 BM3 have been evaluated from *Bacillus megaterium*.

Sawayama et al. (Sawayama et al., 2009) demonstrated a group of variants for cytochrome P450 BM3 from *Bacillus megaterium*. Verapamil and asterimazole metabolites have been produced by P450 BM3 variants.

Weis and coworkers achieved a hydroxylation reaction by using bifunctional cytochrome P450 P450 enzymes (Weis et al., 2009). This biotechnological application included the preparation of metabolites of diclofenac and cloroxazone which are 4'-hydroxydiclofenac and 6-hydroxychloroxazone by biohydroxylation. The mentioned reaction of diclofenac and chloroxazone is shown in Figure 7.

Rinnofner et al. (Rinnofner, Kerschbaumer, Weber, Glieder, & Winkler, 2019) reported the hydroxymetabolites of ibuprofen using *Pichia pastoris* as a catalyst. In this study, the synthesis made in the presence of catalyst and then products were lyophilized. Subsequently preparative LC-MS analysis was performed. The spectral data of the products obtained in this way were compared with the previous studies and the metabolites were given as a percentage. Accordingly, 83% of 502 mg ibuprofen was converted into its metabolites. Of these, 30% is 2-OH ibuprofen, 37% is 1-OH ibuprofen and 16% is an unknown metabolite (Figure 8).

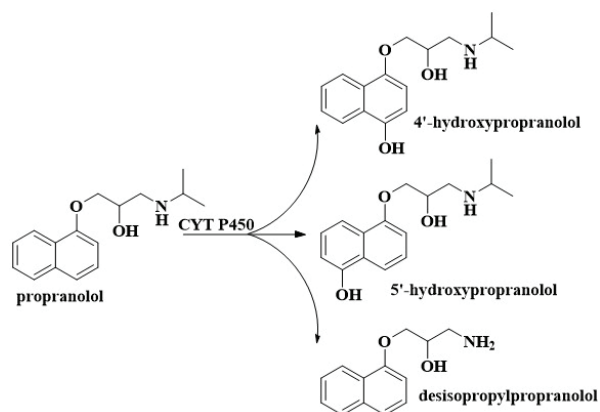


Figure 6. Three metabolites of propranolol formed by cytochrome P450.

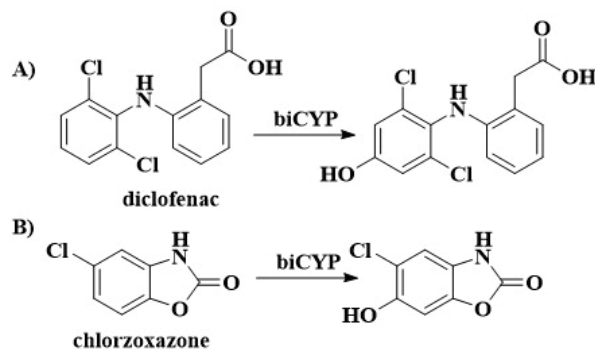


Figure 7. Biohydroxylation of A) diclofenac and B) chloroxazone by bifunctional P450s (biCYPs).

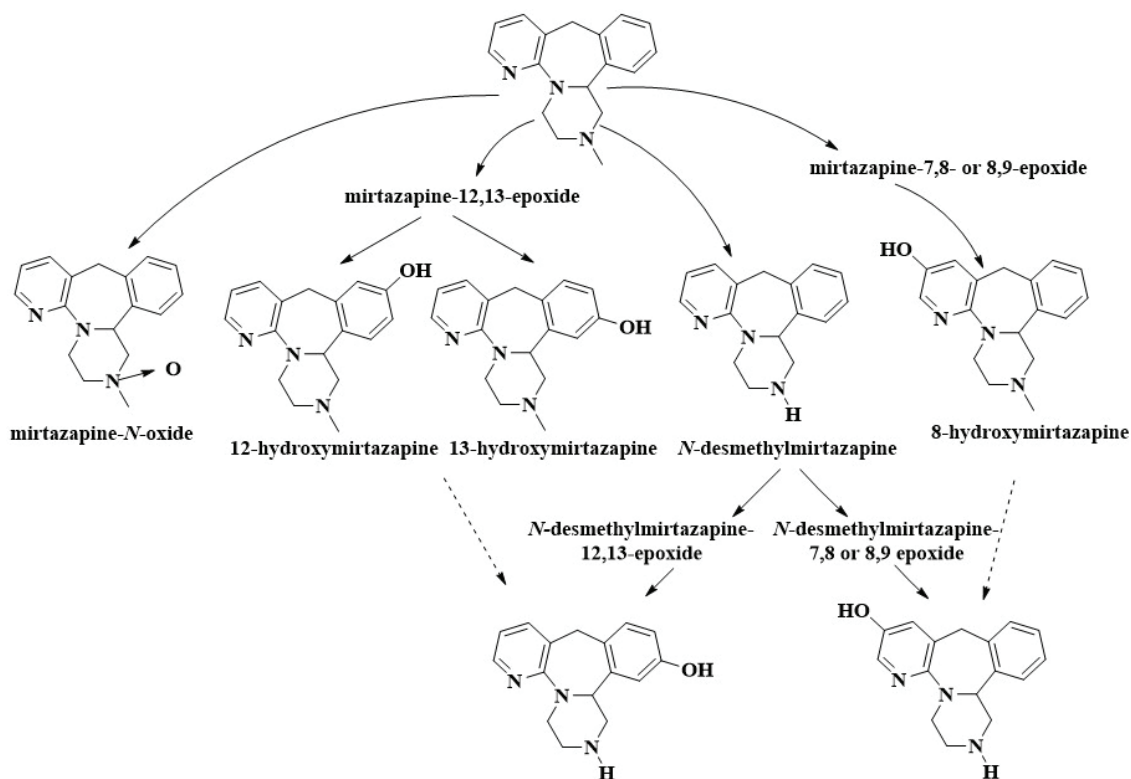


Figure 5. Schematic suggestion for biotransformation of a racemic mixture of mirtazapine to give it all metabolites after incubation with *Cunninghamella elegans* after 168 h.

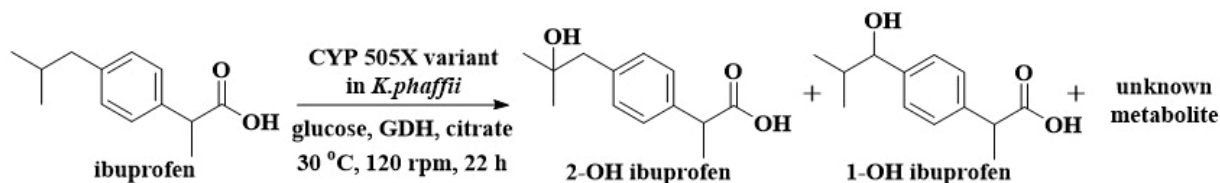


Figure 8. The summarized biooxidation reaction of ibuprofen.

Kuo et al. (Kuo et al., 2004) synthesized various putative Phase I and Phase II duloxetine metabolites. The major metabolite of duloxetine is hydroxylated metabolite in the naphthalene ring. 4/5/6-hydroxyduloxetine (Figure 9) has been synthesized with the same method. The synthesis of other metabolites of duloxetine was given as a reference.

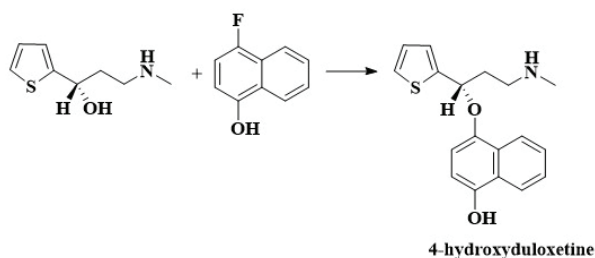


Figure 9. Synthesis and structure of 4-hydroxyduloxetine.

Lombardino reported the synthesis of piroxicam monohydroxylated metabolites using 2-amino-hydroxypyridines (Lombardino, 1981). The multistep pathway is summarized in Figure 10.

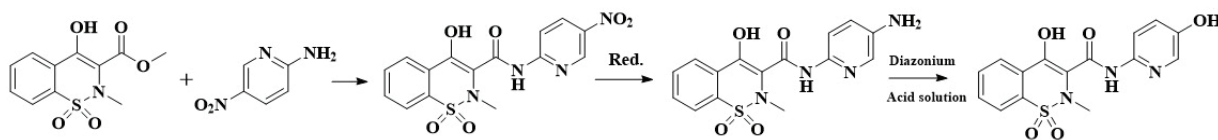


Figure 10. 4-The multistep synthesis pathway for monohydroxylated piroxicam metabolite.

Steinbrecht et al. (Steinbrecht et al., 2020) reported the UPOs for the metabolites synthesis of cytostatic drug cyclophosphamide. 4-Hydroxycyclophosphamide metabolite was performed by a peroxygenase from *Marasimius rotula* (Figure 11).

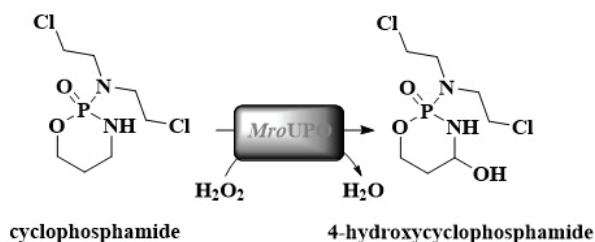


Figure 11. 4-Hydroxycyclophosphamide metabolite of cyclophosphamide obtained by *MroUPO*

The oxidation of amines

As a result of metabolic *N*-dealkylation of secondary aliphatic and alicyclic amines, they produce primary amine and unsub-

stituted alicyclic amine metabolites. The tertiary amines and heterocyclic nitrogen compounds are biotransformed by oxidative dealkylation and *N*-oxidation. Schematic representation of *N*-oxidation of amines is given in Figure 12.

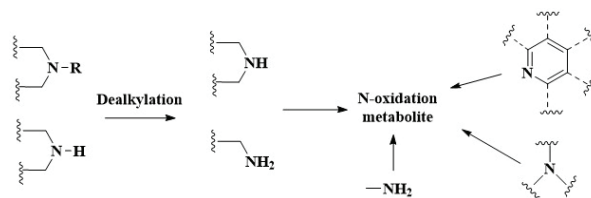


Figure 12. *N*-oxidation of amines.

Sun L. et al. (L. Sun, Huang, Liu, & Zhong, 2004) reported the major metabolites (*N*-demethylation, *O*-demethylation, and sulfate conjugation) of verapamil produced by *Cunninghamella blakesleeana*. Metabolites that are shown in Figure 13 were isolated via the semipreparative liquid chromatography-ion trap mass spectrometry method and identified by ¹H-NMR and ESI-MS analyses.

Fodi et al. (Fodi et al., 2018) reported the biomimetic synthesis of amiodarone metabolites. Metabolite of antiarrhythmic amiodarone and *N*-desmethylamiodarone were obtained by biomimetic oxidation as a major metabolite (Figure 14).

Another method was given for dealkylation by Çoruh. (Çoruh, 2012). As shown in Figure 15, the dealkylation of alkyl substituted 1,2,4-triazolethiones may be carried out with the cyclization of benzoyl substituted acylthiosemicarbazides in alkaline media.

The tertiary amine and heterocyclic nitrogen compound *N*-oxides are synthesized using molecular oxygen or other oxidants such as hydrogen peroxide, *m*-chloroperoxybenzoic acid, magnesium monoperoxyphthalate, 2-sulphonyloxiridines, dioxiran, dimethyl dioxiran, and oxaziridines (Figure 16). Youssif (Youssif, 2001) and Cai et al. (Cai, Sha, Guo, & Pan, 2012) published excellent reviews about tertiary amine *N*-oxides.

Jaworski et al. (Jaworski et al., 1993) synthesized chlorpromazine-*N*-oxide and fluphenazine-*N*-oxide from chlorpromazine

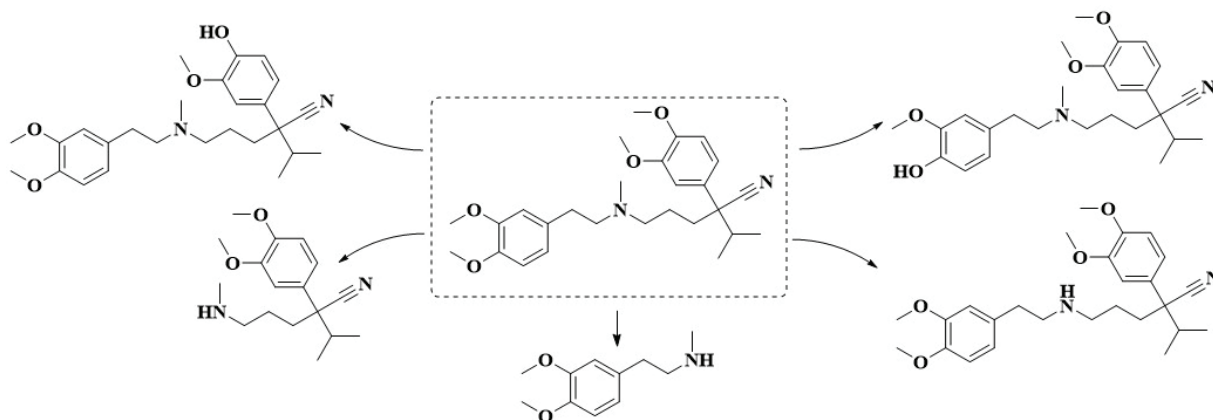


Figure 13. Five major metabolites obtained via transformation of verapamil by *C. blakesleeana*.

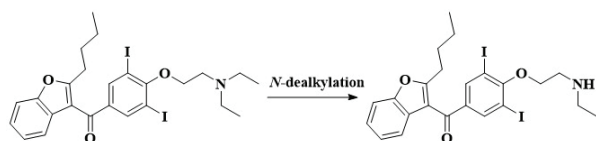


Figure 14. *In vitro* major biotransformation product of amiodarone by using human liver microsomes.

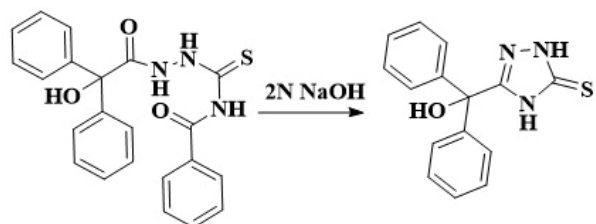


Figure 15. Nonsubstituted 1,2,4-triazole thiones synthesis.

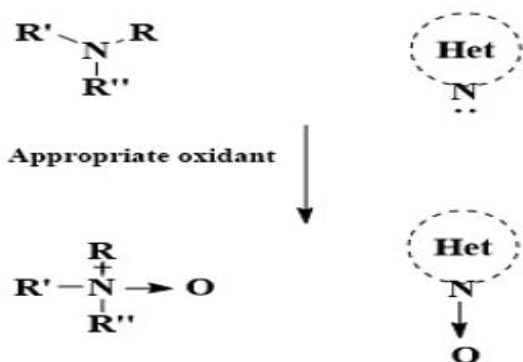


Figure 16. Synthetic N-oxidation of tertiary amines and heterocyclic nitrogen containing compounds.

and fluphenazine in the presence of *m*-chloroperoxybenzoic acid in tetrahydrofuran that yielded 67 % and 45 %, respectively (Figure 17).

Reddy et al. (Reddy, Mukkanti, Kumar, Babu, & Reddy, 2008) reported the synthesis of lansoprazole-N-oxide. As a starting material 2-chloromethyl-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine hydrochloride was used to synthesize lansoprazole *N*-oxide in the presence of *m*-chloroperoxybenzoic acid in chloroform (Figure 18).

Lansoprazole-sulfone-N-oxide was prepared from lansoprazole sulfide in the presence of *m*-chloroperoxybenzoic acid in chloroform (Figure 19).

As shown in Figure 20, Doddaya and Peddakonda reported a synthesis method for chloroquine N-oxide which is a major degradation product of chloroquine and also metabolite of chloroquine (Doddaga & Peddakonda, 2013).

Hanlon et al. (Hanlon et al., 2012) prepared moclobemide N-oxide metabolite (65 mg) by using the FMO enzyme (Figure 21).

The oxidation of thioether

S-dealkylation, desulfuration, and S-oxidation (sulfoxide and sulfone) reactions are known as the metabolic pathways of thioether. A sulfur atom present in the cyclic ring is susceptible to S-oxidation. The sulfoxide functional group containing drugs and metabolites may be further oxidized to a sulfone group. Reddy et al. (Reddy, Mukkanti, Bhaskar, & Reddy, 2008) prepared rabeprazole sulfone from rabeprazole sulfide using *m*-chloroperoxybenzoic acid in a chloroform and methanol mixture (2:1, v/v) at -20 to -25 °C (Figure 22).

Zhang et al. (Zhang et al., 1996) investigated the *in vitro* metabolism of chlorpromazine using *Cunninghamella elegans* (*C. elegans* ATCC 9245). *C. elegans* biotransformed chlorpromazine to its potential metabolites. Chlorpromazine sulfoxide (Figure 23) and other metabolites were characterized by MS, UV, and NMR analyses.

Oxidative O-dealkylation

Oxygen functionality is found in many drugs and other xenobiotics. Drugs containing the ether functional groups are metabolized by oxidative dealkylation.

Antihypertensive prazosin demethylated metabolites have been synthesized by Althuis and Hess (Althuis & Hess, 1977), and the schematic representation of these synthetic pathways are given in Figure 24.

In the concept of drug metabolite synthesis, mammalian cytochrome P450 enzymes have been given great attention in terms of their usage as biocatalysts. Various systems for the heterologous expression of mammalian *cyp* genes have been developed. *Escherichia coli* strains are also used as a host for

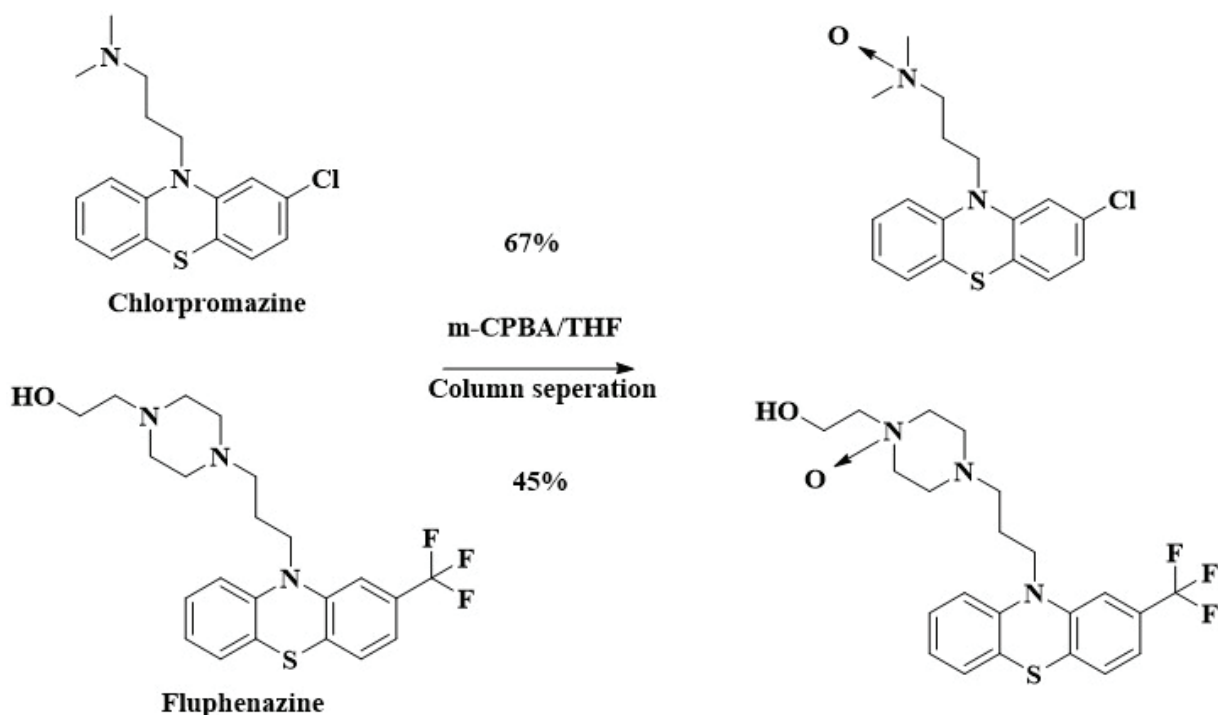


Figure 17. Chemical synthesis of N-oxide metabolites of chlorpromazine and fluphenazine.

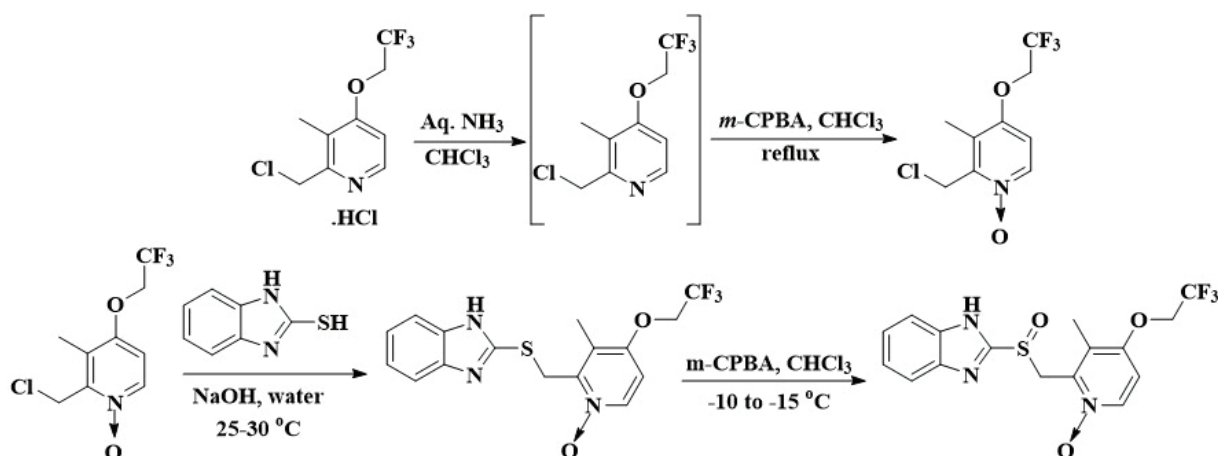


Figure 18. Synthesis protocol for lansoprazole N-oxide.

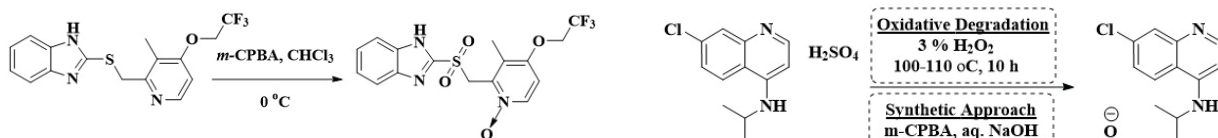


Figure 19. Synthesis protocol for lansoprazole sulfone N-oxide.

drug metabolite synthesis. Particularly, the CYP1A family is an important enzyme for drug metabolite synthesis (Cornelissen, Julsing, Schmid, & Buhler, 2012; Lu et al., 2020). Verapamil (Caswell, O'Neill, Taylor, & Moody, 2013), testosterone (Fessner et al., 2020), lorcaserin (Cusack et al., 2013), NVP-AAG561 (Schroer et al., 2010), diclofenac, diazepam, ibuprofen, phenacetin and cortisol (Winkler, Geier, Hanlon, Nidetzky, & Glieder, 2018), are examples of drugs where metabolites have been synthesized by engineered cytochrome enzymes.

Figure 20. Two proposed synthesis pathways for chloroquine N-oxide.

Extremely selective oxyfunctionalization reactions on drugs including O-dealkylation and hydroxylation can be performed by mimicking the role of the human liver cytochrome P450 monooxygenases and unspecific peroxygenases (UPOs). Gomez de Santoz et al. reported that several UPO variants for their capacity to synthesize human drug metabolites from three pharmaceutical agents: dextro-



Figure 21. Flavin monooxygenase catalyzed *N*-oxidation of moclobemide.

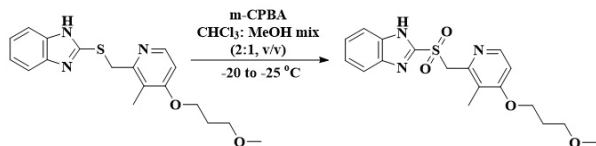


Figure 22. Preparation of rabeprazole sulfone from rabeprazole sulfide.

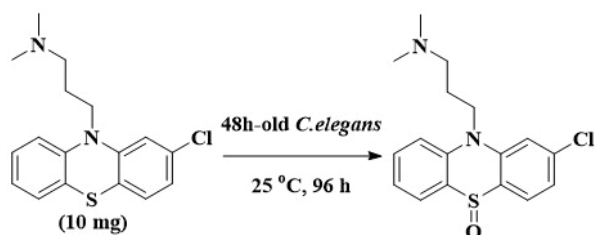


Figure 23. Obtained chlorpromazine sulfoxide metabolite of chlorpromazine after incubation with *C. elegans*.

methorphan (Figure 25), naproxen, and tolbutamide (Gomez de Santos et al., 2019).

Antiinflammatory indomethacin major metabolites are *N*¹-deacyl, *O*-desmethyl, and ester glucuronide analogs. The *O*-demethylated metabolite (1-*p*-chlorobenzoyl-5-hydroxy-2-methyl-3-indolylacetic acid) of indomethacin was synthesized by Strachan et al and the pathway is given in Figure 26 (Strachan, Meisinger, Ruyle, Hirschmann, & Shen, 1964).

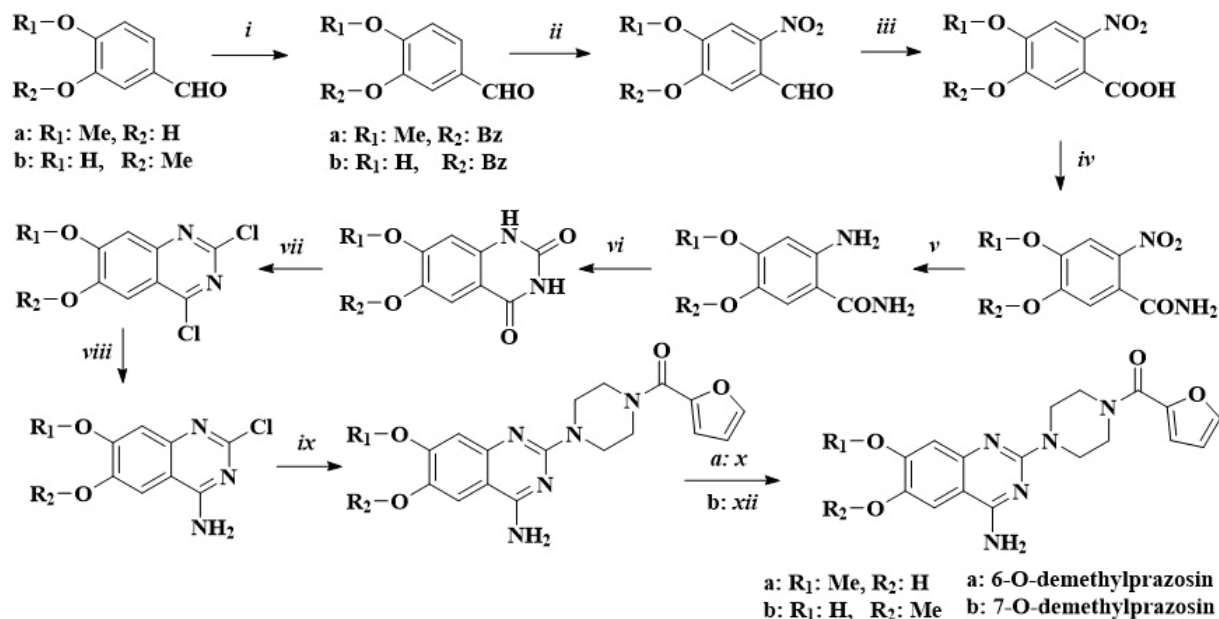


Figure 24. Synthesis of demethyl metabolites of prazosin (i: abs. ethanol, K₂CO₃, benzyl chloride, reflux; ii: 0 °C, HNO₃, 30 °C 15 min; iii: acetone, 10%KMnO₄, iv:SOCl₂ reflux, evap., dioxane, NH₃; v: glacial acetic acid, Fe powder, 90 °C, vi: urea, pyridine in 10%HCl, reflux; vii: POCl₃, *N,N*-dimethylaniline, N₂, reflux; viii: THF, dry NH₃, R.T.; ix: 1-(2-furoyl)piperazin, isoamyl alcohol, reflux; x: CF₃COOH, reflux 2.5h; xi: conc. H₂SO₄, r.t., 0.5h.

Oxidative aromatization

In the literature, metabolic dehydrogenation reaction of some compounds have been reported. Indapamide and nifedipine can be given as examples that can undergo metabolic dehydrogenation. As given in Figure 27, a diuretic drug indapamide dehydrogenation metabolite was synthesized by Sun et al. (H. Sun et al., 2009). Briefly, indapamide has been oxidized with MnO₂ in acetone and then the indolin ring was aromatized to indol.

The cyclic metabolites of drugs

Some drugs can be converted to their cyclic metabolites such as hydralazine and methadone.

The main metabolic route of antihypertensive hydralazine is acetylation. The initially formed *N*-acetyl hydralazine is unstable and cyclizes intramolecularly to form 3-methyl-1,2,4-triazole[3,4-*a*]phtalazine as the major metabolite that was synthesized by Dutkiewicz et al (Figure 28) (Dutkiewicz, Chidan Kumar, Yathirajan, Mayekar, & Kubicki, 2009).

Pohland et al. (Pohland, Boaz, & Sullivan, 1971) synthesized 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-eth-

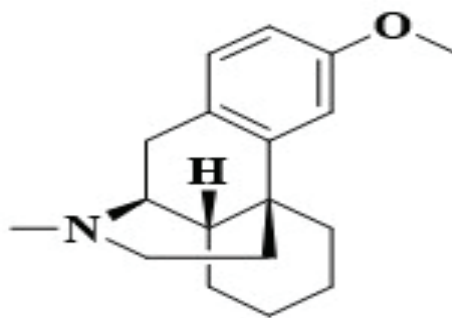


Figure 25. Chemical structure of dextromethorphan.

yl-5-methyl-3,3-diphenylpyrroline, cyclization metabolites of DL-methadone (Figure 29).

Reduction

Reduction reactions play an important role in the biotransformation of many drugs and other xenobiotics containing carbonyl, nitro, and azo groups. N-oxides and sulfoxides are reduced to their corresponding tertiary amines and sulfides. Ketones are reduced to secondary alcohols. Azo compounds are reduced to corresponding amines.

Nitro groups are reduced to amines by many reagents. Drugs containing a nitro group are easily converted to their amine metabolites by the treatment with a suitable reducing agent. Nimesulide is a potent antiinflammatory, antipyretic, and analgesic drug and its amine metabolites can be found in man. Küçük-güzel et al. (Kucukguzel, Kucukguzel, Oral, Sezen, & Rollas, 2005) reported the synthesis of amino nimesulide in rats (Figure 30).

Feely et al. (Feely, Kavanagh, McNamara, & O'Brien, 1999) reported the synthesis of 7-aminoflunitrazepam. 7-aminoflunitrazepam has been readily prepared from the mixture of flunitrazepam and a reducing agent tin (II) chloride dihydrate in ethanol (Figure 31).

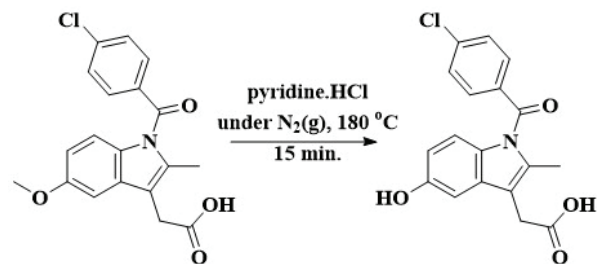


Figure 26. Synthesis of 1-p-chlorobenzoyl-5-hydroxy-2-methyl-3-indolylacetic acid.

Clonazepam is metabolized to 7-aminoclonazepam by nitro reduction via hepatic cytochrome P450. De Paula et al. (de Paula et al., 2015) demonstrated the production of 7-amino-clonazepam metabolite of clonazepam by the microbial transformation (Figure 32).

Azo dyes are used as colorants in the food, drug, and cosmetic industry. The azo compounds are reduced by intestinal anaerobes to toxic amine metabolites. Chung et al. (Chung, Fulk, & Egan, 1978) reported that azo dyes are reduced by intestinal anaerobes. The reduction of product metabolites of drugs and other xenobiotics are commercially available.

Rollas developed a method for the synthesis of aromatic and heteroaromatic amines by reducing azo compounds treatment with hydrazine hydrate without a catalyst (Figure 33). These reactions may be employed for the amine metabolite synthesis from azo compounds (Rollas, 2010).

Hydrolysis

Hydrolysis is a major biotransformation route for drugs containing ester and amide functionality. The metabolic products are carboxylic acids, alcohols, phenols, and amines. Esters are easily converted into their acids and alcohols in the presence of alkaline or acidic medium. The hydrolysis of amides is slower than esters. The hydrolysis product of metabolites of drugs are commercially available.

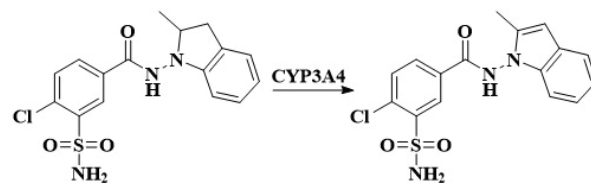
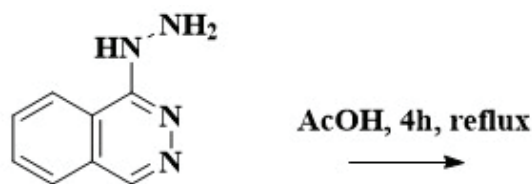


Figure 27. Dehydrogenated metabolite of indapamide.



1-Hydrazinophthalazine

3-Methyl-1,2,4-triazolo[3,4-a]phthalazine monohydrate

Figure 28. Synthesis of the major metabolite of hydralazine.

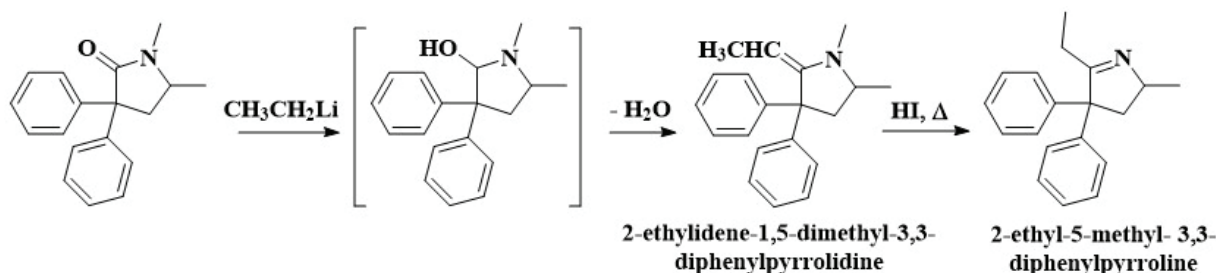


Figure 29. Synthesis of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-ethyl-5-methyl-3,3-diphenylpyrroline.

The synthesis of Phase II metabolites

Major Phase II (conjugative) reactions of the phenolic hydroxyl group are glucuronidation, sulfation, and methylation. Acetylation, methylation, and phosphate conjugation may rarely occur. The carboxyl groups containing drugs and xenobiotics give ester type O-glucuronides (acyl glucuronides) (Baldwin, Robinson, & Williams, 1960). Glucuronide metabolites of resveratrol, flavonoids, morphine, and such phenolic compounds are achieved by silver or borane catalyzed reactions (Stachulski & Meng, 2013). Another conjugative reactions of carboxyl groups are amino acid conjugation. Acyl glucuronides are chemically unstable in an aqueous solution and undergo an intramolecular acyl migration. Therefore, unstable acyl glucuronides are capable of cellular injury such as hepatotoxicity and carcinogenesis (Bailey & Dickinson, 2003). An excellent review about reactivity of acyl glucuronide was published by Bradshaw et al (Bradshaw, Athersuch, Stachulski, & Wilson, 2020). Glutathione conjugation is a formation of a thioether bond by the nucleophilic sulfhydryl group of glutathione.

The synthesis of glucuronide metabolites

The glucuronidation reaction is the most well-known conjugative route in drug metabolism and is catalyzed by the family of uridine diphosphate (UDP) glucuronosyltransferases (UGTs). Metabolites are classified as oxygen, nitrogen, sulfur, or carbon glucuronide according to the heteroatom or carbon attached to the C₁ atom of the glucuronyl group. Conjugation of gluc-

uronic acid occurs on nucleophilic functional groups such as alcohol, phenol, primary, secondary, and tertiary amines, and carboxylic acids, etc. (Argikar, 2012). The most systematic research was initiated in the 1930s by R.T. Williams and collaborators (Pryde & Williams, 1933; Williams, 1938). Phenolic glucuronides have been prepared using glycosyl donors and a chart is given for glucuronic acid donors in Figure 34 (Arewang, Lahmann, Oscarson, & Tiden, 2007).

Yoshimura et al. (Yoshimura, Oguri, & Tsukamoto, 1968) prepared codeine and morphine glucuronides using glycosyl donors (Figure 35).

Acyl glucuronidation is one of the major metabolic pathway of acidic drugs or acidic metabolites that produce by the hydrolysis of ester, amide, and nitrile functional groups or the oxidation of drugs and their metabolites.

The synthesis of naproxen glucuronide conjugate that is shown in Figure 36 was obtained with a 70% yield (Arewang et al., 2007).

O-Glucuronides are generally synthesized by a Koenigs-Knorr reaction (Figure 37). The aglycone reacts with methyl (2,3,4-tri-O-acetyl-1-bromo-1-deoxy- α -D-glucopyran)uronate in the presence of Ag₂CO₃ or Hg(CN)₂ (Kaspersen & Van Boeckel, 1987). Lou et al. (Luo, Hawes, McKay, & Midha, 1992) developed a synthetic method for the quaternary

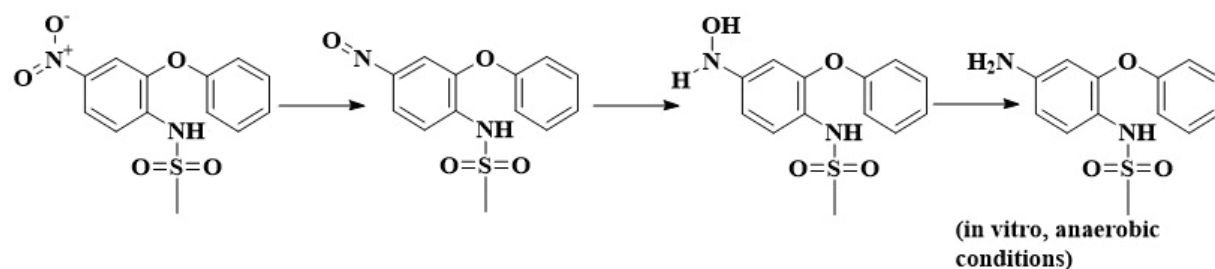


Figure 30. Proposed pathway for the aminonimesulide formation of in vitro metabolite of nimesulide.

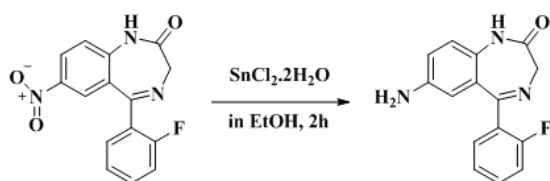


Figure 31. Synthesis of 7-aminoflunitrazepam.

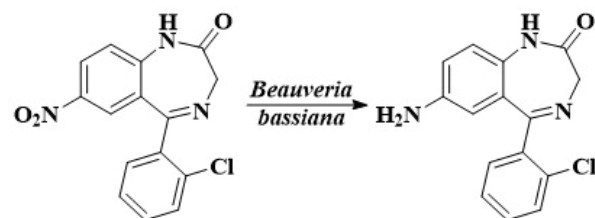


Figure 32. 7-aminoclonazepam metabolite of clonazepam produced by the microbial transformation.

ammonium-linked glucuronide metabolites of the aliphatic tertiary amine group using the same reagent but with NaHCO₃, not silver carbonate.

The synthesis of sulfate metabolites

Sulfate metabolites were prepared using the reaction of drugs containing phenol, alcohol, or amine groups activated by sulphuric acid.

Hydroxyl derivatives and sulphamates (N-sulphates) have been sulfated with SO₃·pyridin complex, SO₃·trimethyl complex, or chlorosulphonic acid (Kaspersen & Van Boeckel, 1987).

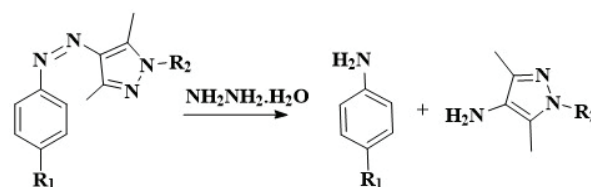


Figure 33. The reduction of azo compounds with hydrazine hydrate.

Foster et al. (B. C. Foster et al., 1991) reported sulfate conjugation of 4'-hydroxyfenazopyridine which is a metabolite of urinary tract analgesic phenazopyridine (Figure 38).

Hoshino et al. (Hoshino et al., 2010) synthesized a sulphate conjugate of resveratrol (Figure 39).

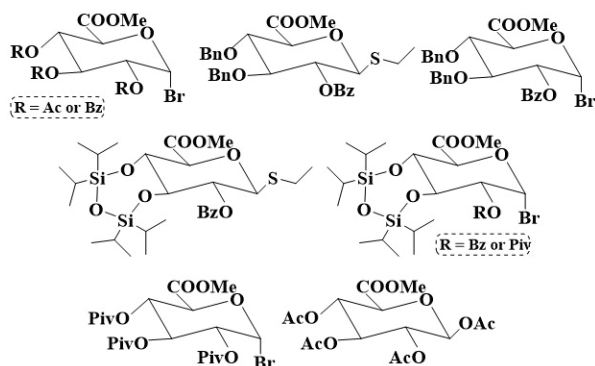


Figure 34. Charts for glucuronic acid donors.

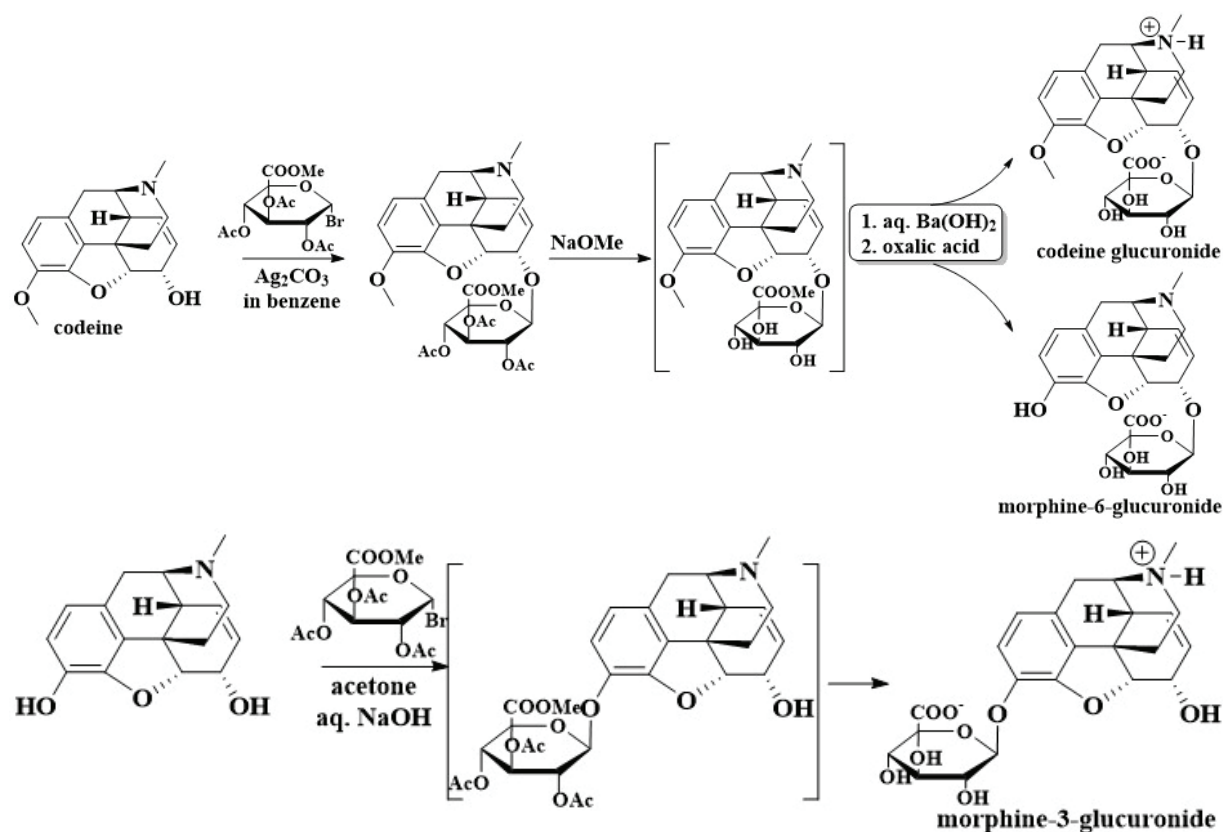


Figure 35. Synthesis of A) codeine glucuronide, morphine-6-glucuronide and B) morphine-3-glucuronide.

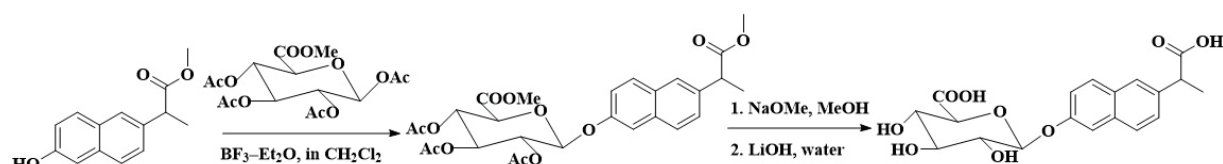


Figure 36. Synthesis pathway for naproxen glucuronide conjugate.

The synthesis of amino acid conjugated metabolites

The major metabolic biotransformations of drugs and other xenobiotic carboxylic acids, with an amino acid or glucuronic acid, are established. Carboxylic acids are mainly converted to glycine conjugates and are rarely converted to glutamine and other amino acid conjugates (Hutt & Caldwell, 1990).

The amino acid conjugation of carboxylic acid was produced from acid chlorides or esters and amino acids. Sinha et al. (Sinha, Praveen, Shrivastava, & Shrivastava, 2012) synthesized amino acid conjugation of valproic acid as prodrugs using thionyl chloride and amino acid esters (Figure 40).

Rasheed et al. (Rasheed, Kumar, Shama, & Mishra, 2011) synthesized amino acid conjugations of aceclofenac as prodrugs using methyl ester of aceclofenac (Figure 41).

The synthesis of glutathione conjugated metabolites

Glutathione conjugation is an important route for the biotransformation of chemically reactive electrophilic drugs, metabolites, and other xenobiotics. Glutathione conjugation is the formation of a thioether bond between an electrophilic center and glutathione (Ketterer & Mulder, 1990).

Huber et al. (Huber, Bartha, Harpaintner, & Schroder, 2009) reported the metabolism of acetaminophen in plant tissues using the cell culture of *Armoracia rusticana* L. as a model system. Acetaminophen glutathione conjugate obtained in the root cells of *A. rusticana* produced a 17% yield.

The synthesis of acetylated metabolites

Acetylation reactions are the metabolic pathways of drugs containing primary aromatic amine, hydrazine, hydrazide, primary aliphatic amine groups, and amine metabolites produced from aryl nitro and azo compounds, such as clonazepam, nitrzepam, and sulfasalazine. Drugs and amine metabolites are generally converted to their acetylated metabolites with acetic anhydride as an acetylating agent.

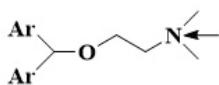
As given in Figure 42, the acetyl-desethyl metabolite of anti-arrhythmic procainamide was synthesized by Adamczyk and Fino (Adamczyk & Fino, 1996).

Nobilis et al. (Nobilis et al., 2006) reported the synthesis of the N-acyl-5-aminosalicylic acid metabolite of mesalazine (Figure 43).

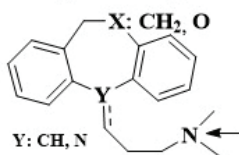
Major metabolites of anticancer aminoglutethimide are acetyl-amino-glutethimide and 5-hydroxylglutethimide. The acetylated metabolite of the N-hydroxy metabolite of aminoglutethimide was synthesized by Foster et al. (A. B. Foster et al., 1984) using pentafluorophenyl acetate as an acetylating agent (Figure 44).

The major metabolite of antitubercular isoniazid is acetyl isoniazid. Fox et al. (Fox & Gibas, 1953) synthesized acetyl isoniazid.

Bicyclic antihistaminic drugs



Tricyclic antidepressants



Antipsychotic agents

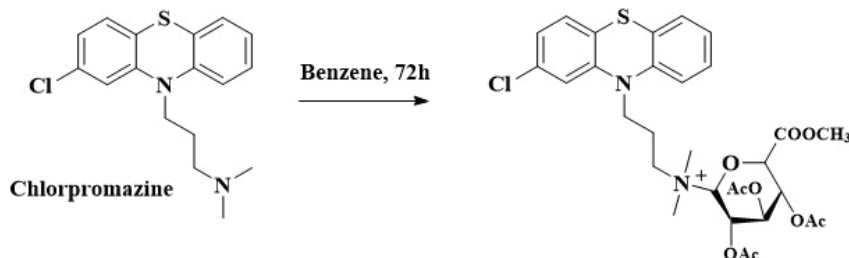
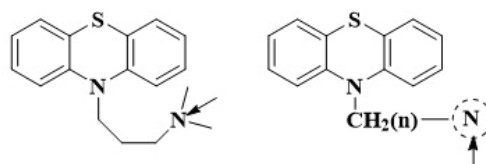


Figure 37. Synthesis of glucuronide metabolites from aliphatic tertiary amines and chlorpromazine by Koenigs-Knorr reaction.

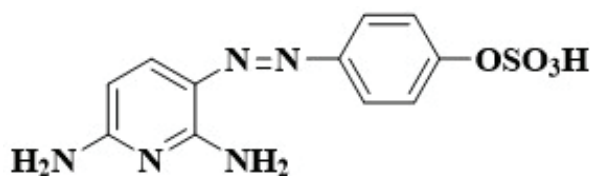


Figure 38. Chemical structure of 4'-SO₃H, O-sulfate monoester of 4'-hydroxyphenazopyridine.

zid from isoniazid, acetic anhydride, and glacial acetic acid (Figure 45).

The synthesis of methylated metabolites

Methylation reactions are a minor pathway for conjugating drugs, xenobiotics, and dietary compounds. O-methylation occurs in the phenolic groups of a variety of endogenous and catecholic compounds (Sang, Lambert, Ho, & Yang, 2011). The methylated drugs are less polar than the substrate. As a

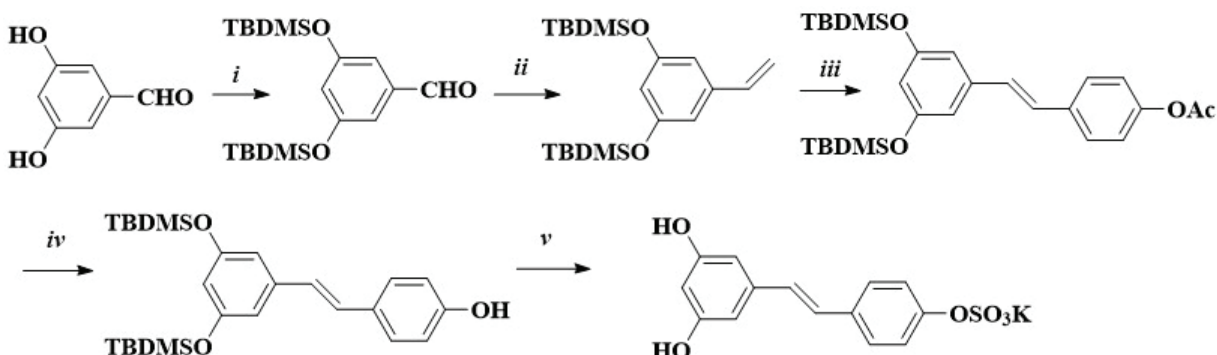


Figure 39. Synthesis of sulfate conjugates of resveratrol (i: TBDMSO, DMF, 80%; ii: NaNH₂, MePPh₃Br, Et₂O, 72%; iii: Pd(OAc)₂, Et₃N, PPh₃, MeCN, 40%; iv: NaOMe, MeOH, 92%; v: SO₃-NMe₃, MeCN, Et₃N; and KF, MeOH/H₂O).

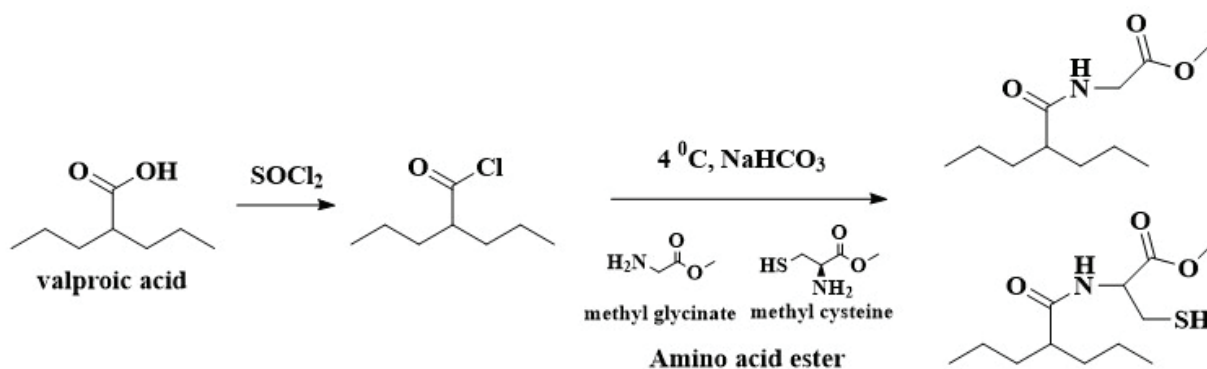


Figure 40. Amino acid conjugate synthesis of valproic acid.

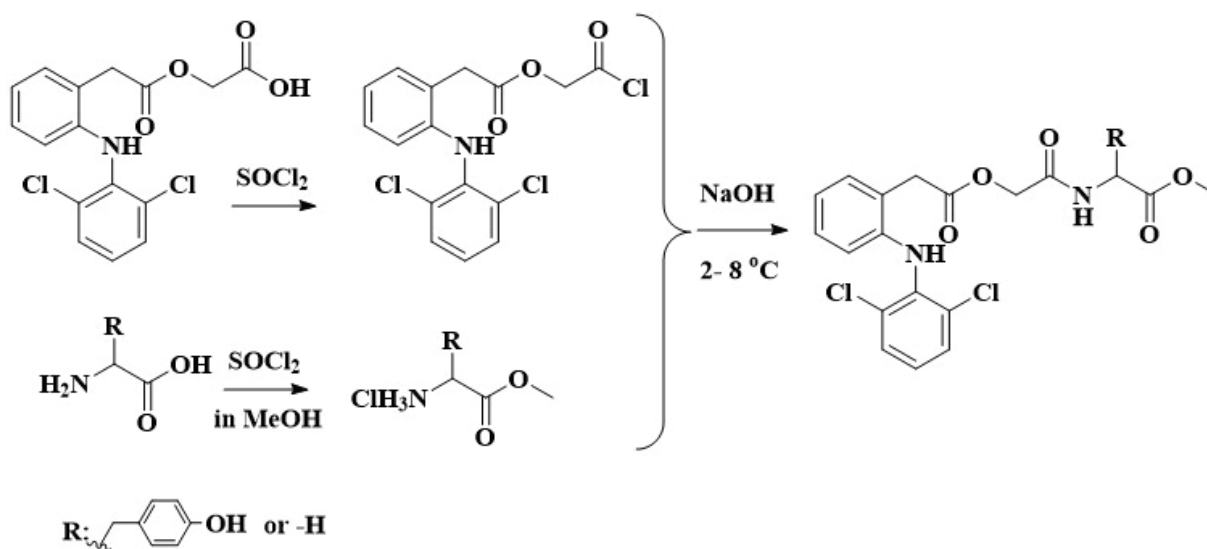


Figure 41. Synthesis of prodrugs of aceclofenac.

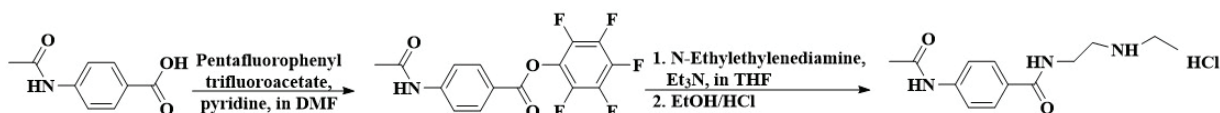


Figure 42. Synthesis of N-acetyl desethylprocainamide.

result of the methylation of morphine codeine was produced (Boerner, 1975). As shown in Figure 46, biogenic amines histamine and norepinephrine were converted to methylated metabolites from their amine groups (Brown, Axelrod, & Tomchick, 1959; Frère & Verly, 1971; Rice, 1977).

The analysis methods of metabolites

For reference standards, the metabolites of drugs and other xenobiotics are used *in vivo* and *in vitro* studies (Sidelmann et al., 1997; Turgeon, Pare, Lalande, Grech-Belanger, & Belanger, 1992; Williams, 1943). In preclinical drug development, the ADMET properties of the potential drug candidates were evaluated in terms of their prospectivity. The essential process in the discovery and development of new drugs is the isolation and identification of their metabolites. The instability in a small number of metabolites in the biological matrix makes isolation and identification difficult. Therefore, one of the best methods is the synthesis of metabolites as a reference standard. The struc-

ture of metabolites has been elucidated using several spectroscopic methods, particularly NMR and MS.

The drug and metabolites analysis is important for pharmacokinetic experiments. Drugs and their metabolite levels are commonly measured using high performance liquid chromatography (HPLC). Mass spectrometry coupled with chromatography and nuclear magnetic resonance spectroscopy (NMR) are widely used techniques for metabolite analysis (Schaber et al., 2001). The mass spectrometer is an important instrument for the identification of metabolites of drugs and other xenobiotics (Constanzer, Chavez-Eng, Fu, Woolf, & Matuszewski, 2005; Nelson, Garland, Breck, & Trager, 1977). Generally, analytes are separated by the suitable Liquid Chromatography (LC) column (Gill, Law, & Gibbs, 1986; Mackichan, 1980). The mass spectrometer is used as a detector. The LC/MS or GC-MS techniques are employed for the isolation and identification of metabolites in biological fluids or tissue extract (Petsalo, Turpeinen, Pelkonen,

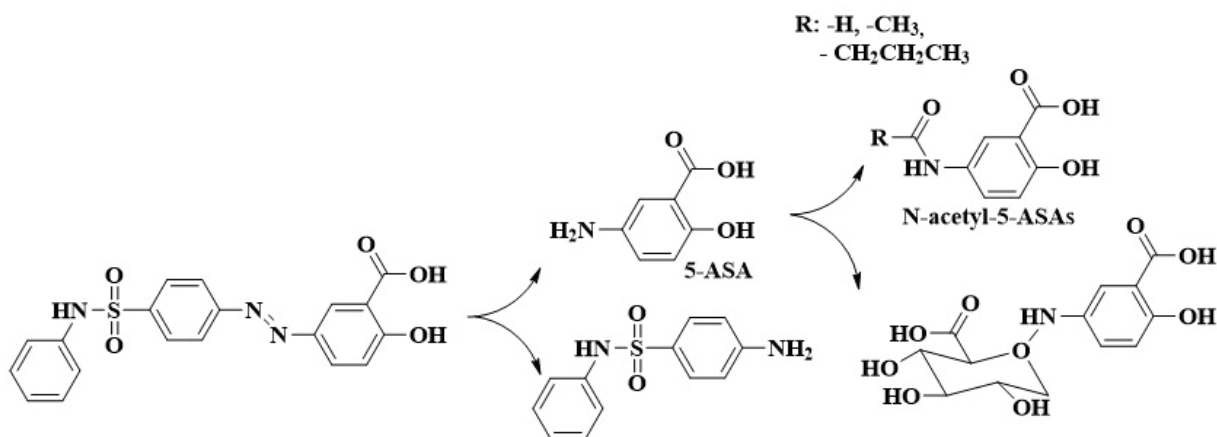


Figure 43. Synthesis of N-acyl-5-aminosalicylic acid metabolite.

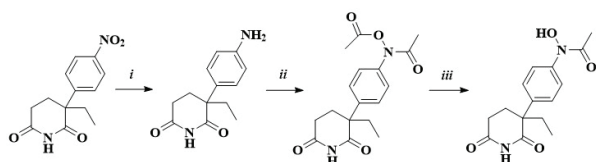


Figure 44. Synthesis of acetylated metabolites of N-hydroxy amino-glutethimide (i: acetone, Zn, NH_4OAc ; ii: $(\text{CH}_3\text{CO})_2\text{O}$; iii: 4M NH_3 , 30 min).

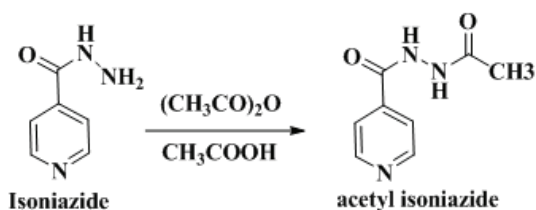


Figure 45. Synthesis of acetylisoniazide.

& Tolonen, 2008; Ren, Zhang, Kong, & Wang, 2018; Wu, Wang, Yang, & Sui, 2019).

Wheals and Jane (Wheals & Jane, 1977) reviewed an analysis of drugs and their metabolites using high-performance liquid chromatography. In this review, the applications of steroids, antibiotics, oxygen-containing compounds, vitamins, alkaloids, nitrogen-containing compounds, prostaglandins, sulfur-containing compounds were demonstrated. The LC-MS/MS technique provides superior selectivity, sensitivity, and analysis for detecting plasma concentration in pharmacokinetics studies and prognosis of acute poisoning. A novel validation method to measure the amount of venlafaxine and its five metabolites by using LC-MS/MS was suggested by Gu et al. (Gu et al., 2018). Michely and Maurer (Michely & Maurer, 2018) reported a fast LC-MS/MS quantification approach for 45 drugs and their relevant metabolites (Fang et al., 2006). Correia et al. (Correia, Rao, Ballet, & Globisch, 2019) demonstrated the combination of untargeted metabolomic analysis and metabolic conversion for the selective detection of glucuronide conjugates by using the UPLC-MS/MS in human urine samples. Ishigai et al. (Ishigai, Langridge, & Bordoli, 2001) studied the dynamics of enzyme-catalyzed glutathione conjugation by electrospray

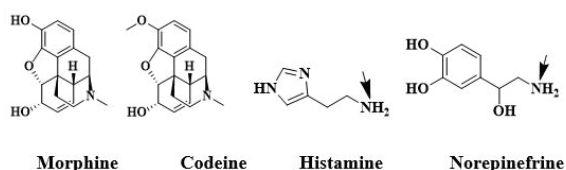


Figure 46. Drugs undergo methylation metabolism.

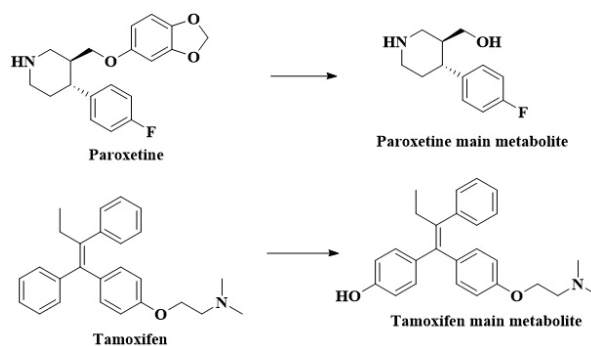


Figure 47. Paroxetine, tamoxifen, and their main metabolites.

quadrupole/time-of-flight (Q-TOF) mass spectrometry with a nanospray interface. The online combination of LC with the inductively coupled plasma (ICP) mass spectrometer offers an excellent method for metabolite identification and structure characterization (Kostiainen et al., 2003). Currently, a variety of techniques are used for the analysis of drug metabolites such as orbitrap technology (OT), high-resolution mass spectrometry (HRMS), high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) high-performance liquid chromatography-inductively coupled plasma tandem mass spectrometry (HPLC-ICP-MS/MS), and hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS). (Cece-Esensenca et al., 2016; Helfer, Michely, Weber, Meyer, & Maurer, 2015; King et al., 2019; Klencsar et al., 2018; Li et al., 2018) Xing et al. (Xing, Zang, Zhang, & Zhu, 2015) presented a new application of high-resolution mass spectrometry (HRMS)-based data-mining tools in tandem to provide a fast and comprehensive metabolite profiling of combination drugs. In his research, a metronidazol-pantoprazole-clarithromycin combination was used as the model.

Sundell et al. (Sundell et al., 2019) developed a LC-MS/MS method for the qualification of rifampicin, isoniazid, pyrazinamide, ethambutol, and their metabolites 25-desacetyl-rifampicin, isonicotinic acid, acetyl isoniazid, and 5-hydroxy pyrazinamide.

Wang et al. (Wang et al., 2019) designed, developed, and validated a rapid, simple, and sensitive method for simultaneous quantitation of four CYP450 probe drugs; phenacetin, omeprazole, metoprolol, midazolam, and their metabolites (acetaminophen, 5'-hydroxyomeprazole, α -hydroxymetoprolol, 1'-hydroxymidazolam) using an ultra high-performance liquid chromatography-tandem mass (UHPLC-MS/MS) spectrometry.

Therapeutic drug monitoring is an important tool for correlating the drug dose to drug and metabolite concentrations in the body and the therapeutic and adverse effects. Protti et al. (Protti et al., 2020) reported an analysis method, a capillary volumetric blood micro sampling, for the selective serotonin reuptake inhibitors fluoxetine and its metabolite, norfluoxetine; sertraline and its metabolite, desmethyl sertraline.

In some cases, the structure of the formed metabolites was not elucidated by the LC/MS technique. An alternative possibility was to isolate the metabolite from the incubation matrix and to elucidate the exact structure using a nuclear magnetic resonance (NMR) method (H. Sun et al., 2009; Zhang et al., 1996).

The non-aqueous capillary zone electrophoresis (NACE) method was used for the analysis of some drugs and their metabolites. Flores et al. (Flores, Nevado, Salcedo, & Diaz, 2004) reported the analysis of paroxetine, tamoxifen, and their main metabolites in urine by NACE (Figure 47).

CONCLUSION

In recent years, discovery and development of new drugs are prerequisite for the evaluation of drug safety and risk assessment. The metabolic profile, metabolite toxicity, metabolite stability, active metabolites pharmacological testing, and pharmacokinetics of a new drug should be defined. Therefore, the synthesis of metabolites is an important area of research for metabolites. Several analytical methods are used for the isolation and detection of metabolites. The LC-MS/MS and LC-MS-NMR systems enable a routine analysis of metabolites.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- S.R.; Data Acquisition- S.R., Z.Ş., P.S.O.Ö.; Data Analysis/Interpretation- S.R., Z.Ş., P.S.O.Ö.; Drafting Manuscript- Z.Ş., P.S.O.Ö.; Critical Revision of Manuscript- Z.Ş., P.S.O.Ö., S.R.; Final Approval and Accountability- Z.Ş., P.S.O.Ö., S.R.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: Authors declared no financial support.

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