

Metabolite detection and profiling using Analytical Methods.

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ABSTRACT:

The mission of pharmaceutical research companies is to develop safe and effective drugs, and bring them to market for the treatment of diseases in the shortest possible time. A few lead compounds are selected for the assessment of safety, and are tested for their absorption (A), distribution (D), metabolism (M), excretion (E) and toxicological (T) properties. These early development phases of ADMET studies are conducted in test systems (in vitro), in living organisms (in vivo) and by computational models (in silico). From the initial stages to final stages of modern drug discovery process, the vital tool for detecting & characterizing metabolites is MS (Mass spectrometer) hyphenated with other techniques. In-vitro methods and cell lines containing heterologously expressed drug metabolizing enzymes are the methods used for generation of metabolites. The use of HPLC-MS/UPLC-MS and high resolution MS, enables to in depth metabolite profiling studies and it may also be possible to characterize and identify the types and site of biotransformation.

KEYWORDS: Forced degradation study, biotransformation, regioselective, preformulation, ICH.

1. INTRODUCTION

The drug discovery phase starts with the identification and validation of the molecular target that is associated with the disease with which the potential drug interacts. High-throughput screening (HTS) is the most common way to test hundreds of thousands of different chemical compounds for their ability to modify the target. A few lead compounds are selected on the basis of ADMET studies that are conducted in vitro (in test systems), in vivo (in living organisms) and by in silico (computational methods).

The drug development process then continues with lead optimization and manufacturing process. The detection and identification of drug metabolites in samples obtained from both in vivo and in vitro studies in preclinical species and then later in clinical phase studies is an important part of discovery process.

These studies will ultimately lead to complete description of metabolism of optimized lead compounds in humans prior to its registration and launch as a new medicine. However, the depth of information required at each stage of drug discovery process varies. In the early stages, the metabolic stability (or lack of it) in a simple in vitro system (provided by hepatic microsomes) is required to be known. With the evolvement of drug discovery process, information on metabolite identification and disposition of lead becomes more assured. Further, it becomes important to know that the lead compounds are undergoing what kind of biotransformation with information on the site(s) of metabolism so that the structures can be optimized to improve factors such as bioavailability and pharmacokinetics.

From the initial stages to final stages of modern drug discovery process, the vital tool for detecting & characterizing metabolites is MS (Mass spectrometer) coupled with HPLC (High-performance liquid chromatography) or UPLC (Ultra high performance liquid chromatography) [1-3].

The speed, selectivity and sensitivity of the LC–MS instruments available for drug metabolism studies have been significantly increased in recent years, enabling higher throughput and faster turn around time.

Also, stress testing (Forced degradation study) is performed to establish intrinsic stability, provide data on degradation product(s) and mechanisms of decomposition of drugs [4]. It is carried out under the conditions of hydrolysis, photolysis, oxidation and dry heat to isolate and characterize degradation product(s), develop and validate stability-indicating assay methods (SIAMs), determination of preformulation and excipient compatibility and for regulatory compliance [5-9]. Presence of linkages like ester, ether, amide and active methylenes in chemical structures of some drugs make them susceptible to cleavage.

Williams et al., (2017) reported a preparative-scale biotransformation using whole yeast cells stably expressing human cytochrome P450 1A1 (CYP1A1) enzyme, that allows regioselective C6-hydroxylation of 5,7-dihydroxyflavone (**chrysin**) to form 5,6,7-trihydroxyflavone (**baicalein**). Optimal aeration, natural pH and maintenance of glucose concentration, throughout the reaction, played very important roles in the biotransformation reaction. This kind of study provides an opportunity for further exploring the utility of stable recombinant CYP enzyme-expressing yeast cells for industrial production of medicinally important polyphenolic compounds [10] (Fig. 1).

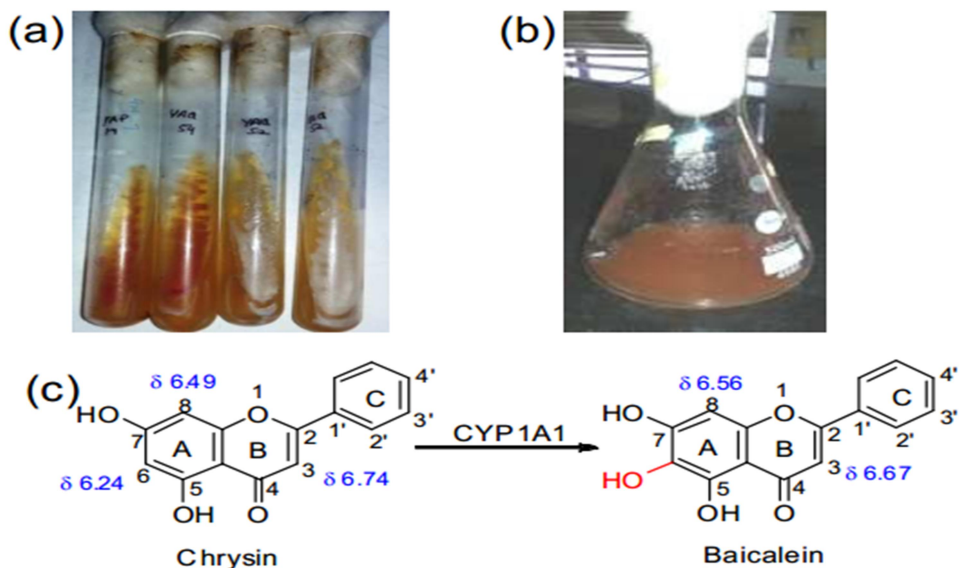


Fig. 1: Biotransformation of chrysin to baicalein

Sordon et al., (2016) subjected natural flavonoids, such as naringenin, hesperetin, chrysin, apigenin, luteolin, quercetin, epicatechin and biochanin A to microbiological transformations by *Rhodotorula glutinis* (Fig. 2). Yeast was able to regioselective C-8 hydroxylation of hesperetin, luteolin and chrysin. Naringenin was transformed to 8- and 6-hydroxyderivatives. Quercetin, epicatechin and biochanin A did not undergo biotransformation. A metabolic pathway for the degradation of chrysin was also elucidated. The metabolism of chrysin proceeds via an initial C-8 hydroxylation to norwogonin, followed by A-ring cleavage to 4-hydroxy-6-phenyl-2H-pyran-2-one [11].

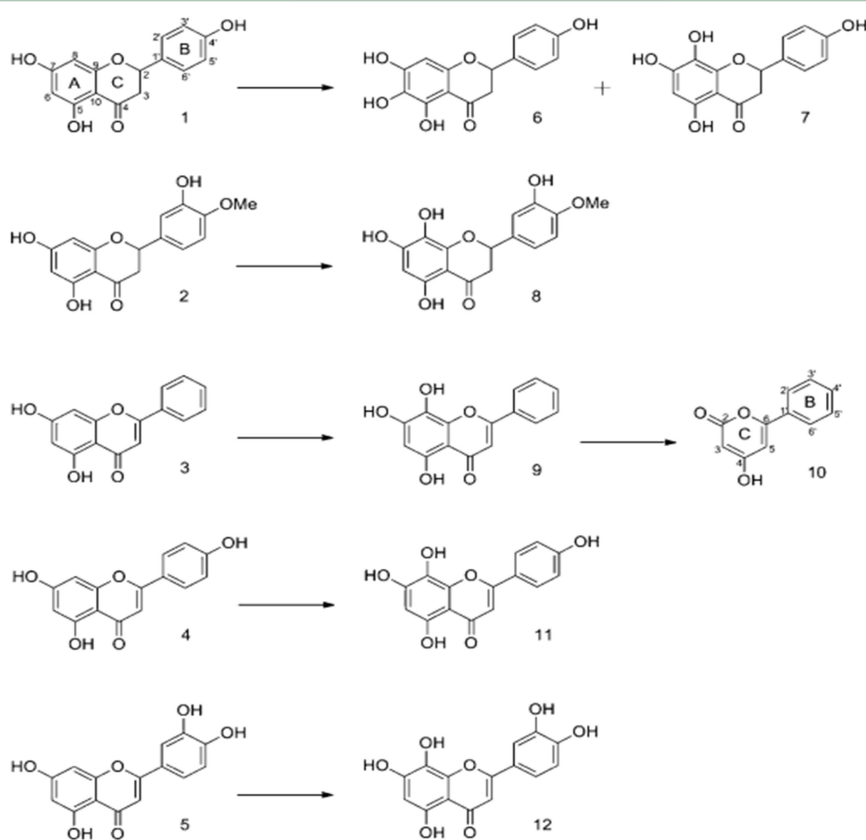


Fig. 2: Transformation of naringenin (1), hesperetin (2) chrysin (3) apigenin (4) luteolin by *Rhodotorula glutinis*

Kinne et al., (2009) did regioselective preparation of 5-hydroxypropranolol and 4'-hydroxydiclofenac with a fungal peroxygenase. Their study raise the possibility that fungal peroxygenases may be useful for versatile, cost-effective, and scalable syntheses of drug metabolites [12].

Park et al., (2008) conducted a study on atorvastatin in which two metabolites of atorvastatin, para- and ortho-hydroxyatorvastatin were produced by human liver microsomes and human recombinant CYP3A enzymes, and the enzyme kinetic pattern exhibited substrate inhibition. The intrinsic clearance rate of their study suggested CYP3A4 is the major P450 isoform responsible for atorvastatin metabolism [13] (Fig. 3).

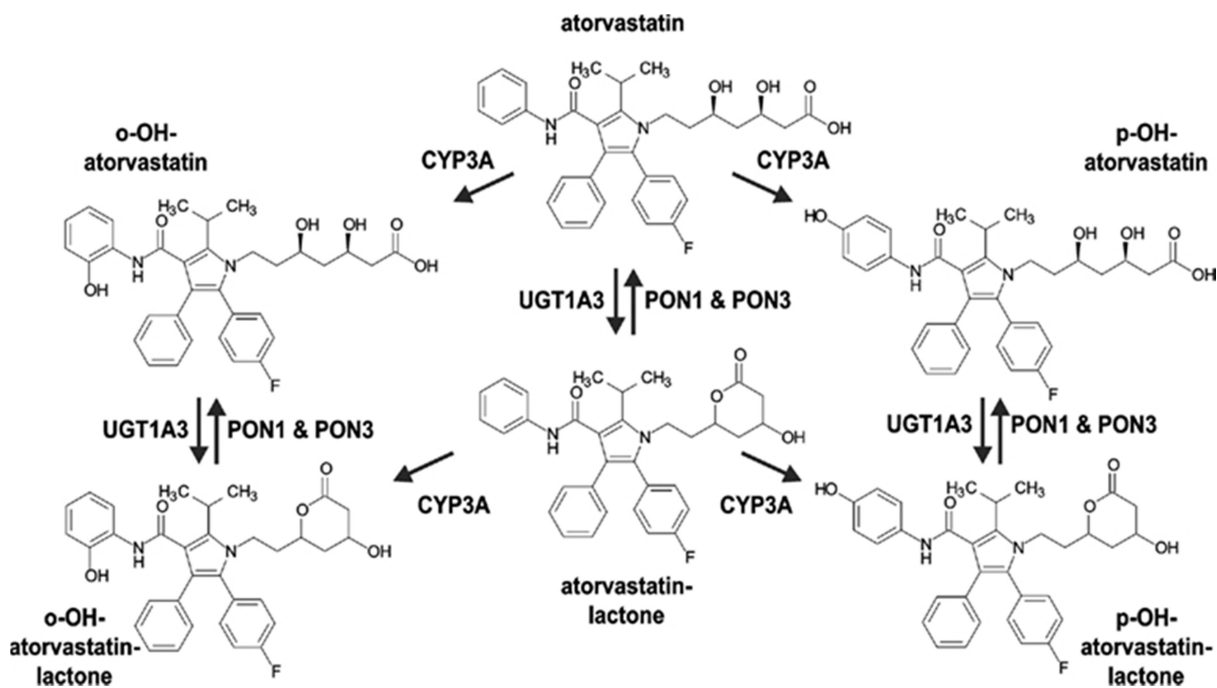


Fig. 3: Metabolism of atorvastatin

Guaratini et al. (2014) performed *in vitro* metabolism of erythraline, the major spirocyclic alkaloid of *Erythrina verna* by biomimetic phase I reactions using Jacobsen catalyst. This produced oxidative metabolites and one metabolite was isolated and identified as 8-oxo-erythraline. This was then evaluated against cancer cells, as HL-60 (promyelocytic leukemia), SF-295 (Glioblastoma) and OVCAR-8 (Ovarian carcinoma) but showed no important cytotoxic activity against tumour cells [14] (Fig. 4).

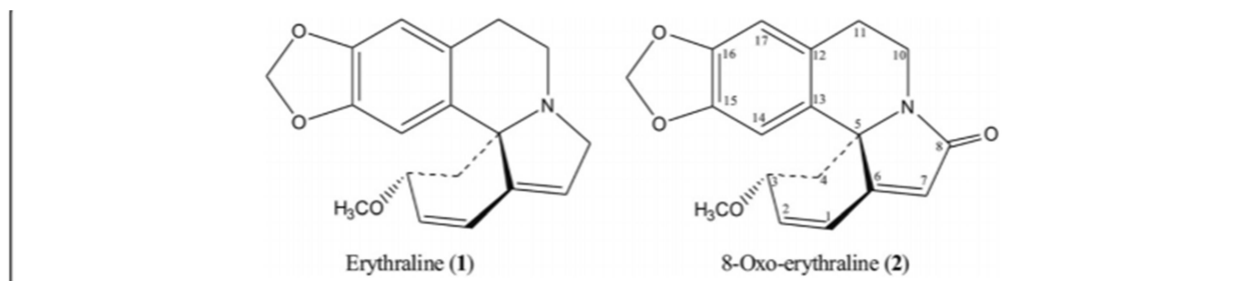


Fig. 4: Structures of erythraline (1) and metabolite (2)

Obach et al., (2005) conducted oxidative and conjugative metabolism of sertraline *in vitro* to identify the enzymes involved in the generation of N-desmethyl, deaminated and N-carbamoyl-glucuronidated metabolites in humans. Using P450 isoform-selective inhibitors and recombinant heterologously expressed enzymes, it was demonstrated that several P450 enzymes catalyzed sertraline N-demethylation with CYP2B6 contributing the greatest extent. For deamination, data supported a role for CYP3A4 and CYP2C19 [15] (Fig. 5).

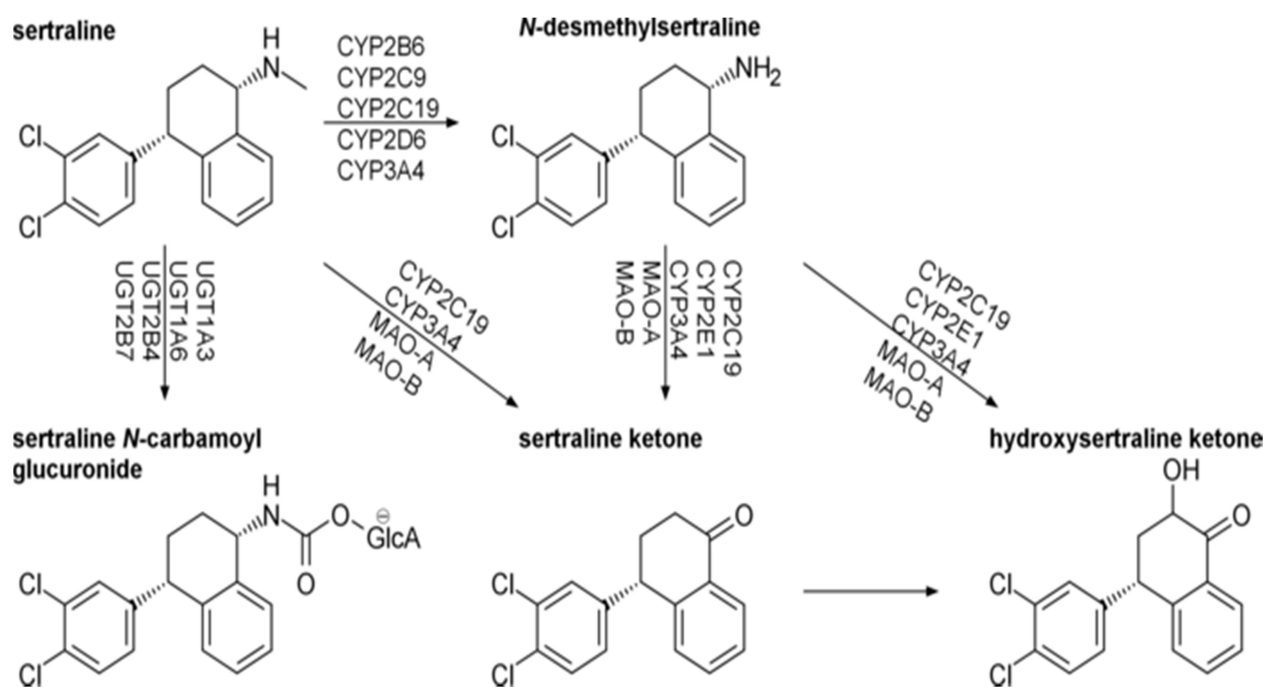


Fig. 5: Metabolism of sertraline

Malgorzata SM and Boguslaw B (2016) studied *in vitro* metabolism of selected antibiotic drugs viz., ciprofloxacin, cefotaxime, fluconazole, gentamicin, clindamycin, linezolid and metronidazole using human liver microsomes. A total of 11 potential metabolites and their

proposed structures were characterized during the incubation of human liver microsomes by comparing their retention times and spectral patterns with those of the parent drug [16].

A degradation product is an impurity which is formed as a result of chemical change in the parent drug during manufacture and storage of drug substance or product due to temperature, light, pH, and water, reaction with container-closure and reaction with an excipient of formulation. The concentration of active ingredients and impurities or degradation products which appear as a result of aging are employed for establishing shelf-life of a drug [17]. Various regulatory authorities including ICH, FDA, CPMP and others require use of SIAM for stability testing of drug substances or product. Further, the degradation products appearing during manufacturing and stability studies are required to be reported in the dossier submitted for product registration (ICH Q3B(R), 2003). Hence, the ICH guideline Q1A(R2) (2003) require forced degradation study on drug substances to provide data on decomposition products, which can be used to establish degradation pathways, intrinsic stability of the molecule and validation of SIAM (Q1A (R2),2003). Forced degradation study is different from accelerated stability testing as the former is conducted under much severe conditions than the later. It does not stick to recommend storage conditions and do not even necessarily predict shelf life. On the contrary, accelerated stability study generates information regarding what may happen during storage under normal conditions. Forced degradation under harsh conditions may create decomposition products which are not commonly formed under long term stability or accelerated conditions [9]. The forced degradation study evaluates susceptibility of drug to hydrolysis over a wide range of pH oxidation and dry heat in solution or suspension. It is to be conducted on a single batch of the drug substance (ICH Q1A (R2), 2003). Additionally, ICH guidelines Q1B (1996) recommend photostability testing of the drug substances and products [18].

Oliverira et al. (2013) did degradation kinetics of atorvastatin under stressed conditions and chemical analysis by HPLC. Atorvastatin has benn found to degrade under acid and basic conditions, including a first order kinetic degradation under basic conditions, which tends to be less stable when studied within acid medium [19].

Vukkum et al., (2012) developed a rapid, reversed phase liquid chromatographic method for the quantitative dtermination of atorvastatin calcium, its related substance and degradation impurities in bulk drugs. The drug substance was subjected to stress studies such as hydrolysis,

oxidation, photolysis and thermal degradation and considerable degradation was observed in acidic hydrolysis, oxidative, thermal and photolytic stress conditions. The formed degradation products were reported and were well resolved from atorvastatin and its related substance. The stressed samples were quantified against a qualified reference standard and the mass balance was found to be close to 99.5 % w/w when the response of the degradant was considered to be equal to the analyte (i.e., atorvastatin), which demonstrates stability-indicating capability of the method. The method was validated in agreement with ICH requirement [20].

2. METHODS FOR GENERATION OF METABOLITES

2.1 *In vitro* methods

Subcellular fractions: Subcellular fractions prepared from organs expressing drug metabolizing enzymes include the cytosolic fraction, the S9 fraction and microsomes. Organs such as intestine, liver, kidney, lung, and skin are known to mediate xenobiotic metabolism. Liver is the major site of drug metabolism. As a result, liver subcellular fractions are often employed for studying metabolic reactions and generation of metabolites. Subcellular fractions can be used to prepare metabolites formed by a number of enzymes such as CYP, FMO, myeloperoxidase, ketoreductase, alcohol dehydrogenase, sulfotransferase, etc [21].

2.1.1 Primary cell-based systems: Intact cells such as primary hepatocytes, contain both soluble and membrane-bound enzymes including the relevant cofactors at or near the appropriate physiological concentrations. As a result, they have greater physiological relevance and can mediate both phase I and phase II metabolism. Cryopreserved hepatocytes are now available for convenient use, and the hepatocytes can be pooled together from different donors. Immortalized human liver cell lines in which enzymes such as CYPs are individually expressed can also be used for relatively large-scale generation of metabolites. Tissue slices such as liver slices contain both phase I and II drug metabolizing enzymes can also be used to generate sufficient quantities of metabolites [22].

2.1.2 Cell lines containing heterologously expressed drug metabolizing enzymes: Several cellular systems that express high levels of CYP enzyme have been used by Rushmore et al. as “CYP bioreactors” for rapid and largescale biosynthesis of metabolites. These include

baculovirus-infected insect cells, immortalized human liver epithelial cells, and E.coli that have been engineered to express large quantities of individual CYP enzymes [23, 24].

3. METABOLITE PROFILING BY ANALYTICAL METHODS

In the initial stages of drug discovery, often before the selection of the series of compounds for further optimization, some idea of metabolic or chemical stability is required. Metabolic stability properties can often be investigated by monitoring the disappearance of parent compound from the reaction incubation mixture. A simple direct infusion of sample into mass spectrometer is required. The methodology is readily automated including MS optimization, etc [25-27].

In the case of chromatographic properties, it is rational “rule of thumb” to assume that most metabolites will be more polar than the parent compound and so can be expected to elute earlier than parent compound. Therefore, it is necessary to ensure sufficient retention of the parent compound. With the evolution of drug discovery process, information on metabolite identification & disposition of lead becomes more assured. Further, it becomes important to know that the type of biotransformation the lead compounds are undergoing with information on \site(s) of metabolism so that the structures can be optimized to improve factors such as bioavailability and pharmacokinetics [28-29].

From the initial stages to final stages of modern drug discovery process, the vital tool for detecting & characterizing metabolites is MS combined with High performance liquid chromatography (HPLC) or Ultra high performance liquid chromatography (UPLC). LC combined with ultraviolet (UV) and thin layer chromatography (TLC) can also be used. This can be attained by adjusting the solvent gradient conditions such that the compound under analysis elutes towards the end of chromatographic run [30].

Though in-vitro investigations are a very powerful means of obtaining metabolic information, there will come the need to assure the presence of in-vitro metabolite in vivo in preclinical studies using species such as dogs and rodents and ultimately the confirmation is done in humans after the initial phase I clinical studies [31].

An essential division of in-vitro metabolite profiling studies involves the detection of reactive metabolites which forms part of risk assessment of potential drug candidates [32]. Metabolism

that results in the generation of reactive metabolites is to be avoided because this property represents a concern for toxicity. Reactive metabolites are unstable by their nature & thus are difficult to detect. Thus, LC-MS based assays may be used to detect these types of metabolites [33, 34].

4. CONCLUSION

The use of HPLC-MS/UPLC-MS and high resolution MS, enables to in depth metabolite profiling studies, it may also be possible to characterize and identify the types and site of biotransformation. Reactive metabolites are also detected by LC-MS. LC-MS in combination with other techniques such as radiolabelled compounds and NMR spectroscopy is thus used for comprehensive metabolic profile and structure elucidation.

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