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Review

Strategies for metabolite profiling based on liquid chromatography

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ABSTRACT

This paper aims at covering the principal strategies based on liquid chromatography (LC) for metabolite profiling in the field of drug discovery and development. The identification of metabolites generated in the organism is an important task during the early stages of preclinical research to define the most proper strategy for optimizing, adjusting metabolic clearance and minimizing bioactivation. An early assessment of the metabolite profile may be critical since metabolites can contribute to pharmacological and/or toxicological effects. The study of metabolites first involves their synthesis/generation and their further characterization and structural elucidation. For such a purpose, both *in vitro* and *in vivo* methods are commonly used for the generation of the corresponding metabolites. Next, analytical methods are used to tackle identification and characterization studies. Among the arsenal of techniques available in our labs, we will focus on LC, especially coupled to mass spectrometry (LC–MS), as one of the most powerful approaches for metabolite identification, characterization and quantification. Here, the topic of metabolite profiling based on LC will be addressed and representative examples of different possibilities will be discussed.

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1. Introduction

The vast majority of drugs are transformed total or partially in the organism. Generally, such processes involve the formation of more polar species than the original drug to be more easily excreted. Besides, these processes commonly imply a loss of pharmacological activity together with a reduction of toxicity. Of course, exceptions to these general patterns sometimes occur in which some drugs may generate pharmacologically active or, even, toxic metabolites. For instance, enrofloxacin is a quinolone antibiotic whose main metabolite ciprofloxacin maintains a high antibacterial effect [1]. The most exceptional cases of metabolic activation deal with the so-called prodrugs, *i.e.* originally inactive molecules that acquire their activity after undergoing metabolism. As a different point, the occurrence of toxicity induced by metabolites is another occasional (and unwanted) phenomenon associated to drug metabolism. As a result, dozens of potentially active molecules must be discarded during the early stages of research and development of new drug candidates due to such problems. Toxicity may ever be detected in approved drugs that are afterwards withdrawn from the market or subjected to restrictions because of unwanted effects often caused by reactive metabolites [2]. Most of the adverse effects linked to metabolites are dose-dependent and can be assessed from regulatory animal toxicity studies *via*

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in vitro and *in vivo* assays. In a few number of cases, however, idiosyncratic adverse effects in humans may occur which are seldom predictable from assays with toxicological species [3]. In these circumstances, activity/toxicity effects of some drugs might differ significantly among patients due to inter-individual variabilities. New promising trends to deal with such differences rely on personalized treatments specifically designed for each patient. Among other possibilities, monitoring drug or metabolite levels in body fluids and designing controlled drug release systems may result in highly efficient strategies to minimize toxicities.

The metabolic biotransformations can be classified into phase I and phase II reactions as follows. Phase I metabolism (or functionalization reactions) introduce functional groups in the structure of the drug. The most common phase I reactions consist of oxidations (aliphatic and aromatic hydroxylation, N-, O- and S-dealkyilation, oxidative deamination, N-oxidation, *etc.*), reductions (azo-reduction and nitro-reduction) and hydrolysis (of esters and amides). Phase II metabolism (or conjugative reactions) consist of the formation of covalent bonds between a reactive functional group of the raw drug or a phase I metabolite with endogenous compounds. Some important phase II reactions comprise glucuronidation, glycosylation, acylation, acetylation, sulfation, methylation and conjugation with amino acids, glutathione (GSH) and fatty acids.

The identification and quantification of metabolites generated in the organism are an important issue in drug discovery and development. The preliminary evaluation of the metabolic profile is a necessary step to define the most proper strategy of drug optimization, adjusting the metabolic clearance and controlling the bio(de)activation. A comprehensive preclinical evaluation, including inter-species comparison may be important to assess the pharmacological and/or toxicological effects of metabolites. Anyway, it should be mentioned that metabolic profiles may differ notably depending on the species assayed. In this way, the variety of metabolites, the concentration levels and kinetics are often species-dependent [4]. In general, the incidence of metabolites on the drug activity or toxicity may be negligible if the metabolism rate is low. It is accepted that metabolites representing less than 10% of the parent systemic exposure in humans may be disregarded. Conversely, those metabolites occurring at concentrations above 10% have to be evaluated more thoroughly to assess their potential effects and risks. If human exposure to these metabolites is similar or lower to that observed in toxicological animal species, no further preclinical tests are needed. However, special attention should be paid to those metabolites present at disproportionately lower levels in animal studies compared to humans [5].

A general scheme to be followed for metabolite profiling in the research and development of new drugs is shown in Fig. 1. The comprehensive evaluation of the drug metabolism comprises various main stages, namely: metabolite generation, isolation and purification, separation, identification and structural elucidation and quantification.

According to Fig. 1, the study of drug metabolism first involves the (bio)synthesis or generation of metabolites for their subsequent characterization and structural elucidation. Preliminarily, *in vitro* assays with model systems of different complexity, such as microsomes, cells and tissues, can be conducted to obtain a first insight on the metabolite formation [6,7]. Electrochemical generation methods have recently been introduced to provide a straightforward and cheap picture of oxidative routes [8,9]. Anyway, *in vivo* assays are required for a more comprehensive and accurate evaluation of the overall drug metabolism. It should be mentioned that apart from experimental approaches, *in silico* methods based on prediction rules are increasingly used for a rough approximation to drug metabolism in order to quickly detect some potential sources of warnings [10,11].

Once metabolites have been generated, they need to be identified and quantified using the great arsenal of analytical techniques available in our laboratories. Often, preliminary sample treatments are applied to purify and preconcentrate the analytes based on protein removal, liquid and solid phase (micro)extraction, *etc.* The resulting extracts are then analyzed using powerful instrumental techniques such as liquid chromatography (LC) coupled to mass spectrometry (MS) and nuclear magnetic resonance (NMR) for performing separation, structural elucidation and quantification of the components of interest. Structural information of metabolites is essential in drug discovery to quickly identify and establish the metabolite profiles and the rate of drug biotransformation. However, metabolite quantification may result in a complex task as standards of most of metabolites are unavailable. This issue may be solved from the estimation of the drug decay or under the assumption of similar instrumental sensitivities for the parent drug and its metabolites. This is true in the case of radioactivity detection and is commonly accepted in the case of UV detection as the

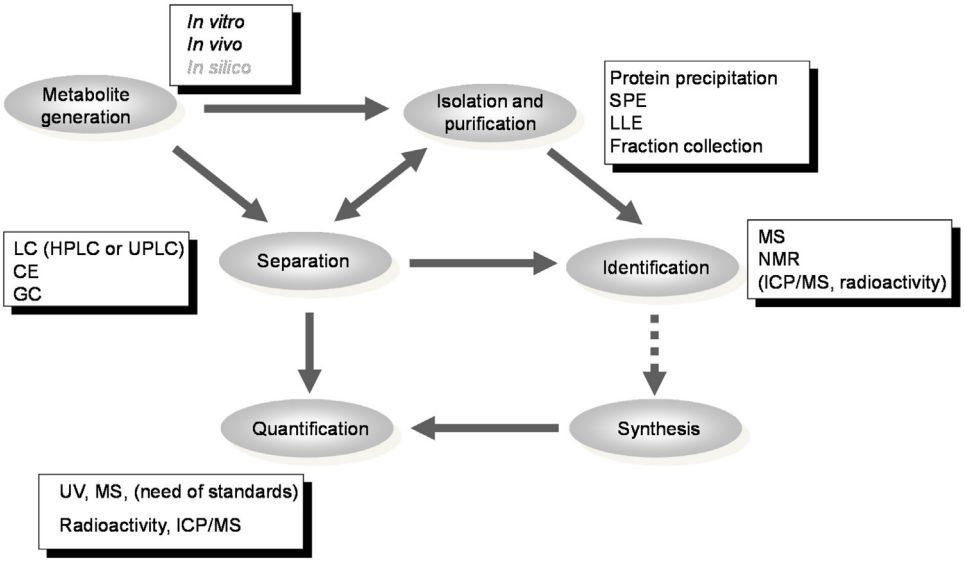

Fig. 1. General strategy for metabolite profiling.