PHARMACOKINETICS, PHARMACODYNAMICS AND DRUG METABOLISM

In Vitro Assessment of Cytochrome P450 Inhibition: Strategies for Increasing LC/MS-Based Assay Throughput Using a One-Point IC_{50} Method and Multiplexing High-Performance Liquid Chromatography

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ABSTRACT: A fast and robust LC/MS-based cytochrome P450 (CYP) inhibition assay, using human liver microsomes, has been fully developed and validated for the major human liver CYPs. Probe substrates were phenacetin, diclofenac, S-mephenytoin, and dextromethorphan for CYP1A2, CYP2C9, CYP2C19, and CYP2D6, respectively. Midazolam and testosterone were chosen for CYP3A4. Furafylline, sulfaphenazole, tranylcypromine, quinidine, and ketoconazole were identified as positive control inhibitors for CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, respectively. To increase the throughput of the assay, a one-point method was developed, using data from CYP inhibition assays conducted at one concentration (i.e., 10 μM), to estimate the drug concentration at which the metabolism of the CYP probe substrate was reduced by 50% (IC_{50}). The IC_{50} values from the one-point assay were validated by correlating the results with IC_{50} values that were obtained with a traditional eight-point concentration–response curve. Good correlation was achieved with the slopes of the trendlines between 0.95 and 1.02 and with R^2 between 0.77 and 1.0. Throughput was increased twofold by using a Cohesive multiplexing high-performance liquid chromatography system. The one-point IC_{50} estimate is useful for initial compound screening, while the full concentration–response IC_{50} method provides detailed CYP inhibition data for later stages of drug development. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:2485–2493, 2007

Keywords: cytochrome P450; drug–drug interactions; drug metabolizing enzymes; high-throughput technologies; human liver microsomes; inhibition; mass spectrometry; mathematical model; one-point IC_{50}; probe substrates

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INTRODUCTION

Cytochrome P4501 (CYP142), CYP2C9, CYP2C19, CYP2D6, and CYP3A4 are important human drug

metabolizing enzymes (DMEs) and are responsible for the biotransformation of greater than 80% of drugs currently on the market.[1] Inhibition of these DMEs could potentially result in a drug-drug interaction (DDI) in patients when concomitant medications are metabolized through similar metabolic pathways.[2,3] This DDI could lead to severe side effects and has resulted in early termination of development, refusal of approval, severe prescribing restrictions, and even withdrawal of drugs from the market. Consequently, *in vitro* CYP inhibition assays are becoming increasingly important in profiling a clinical drug candidate in terms of its potential for a DDI.[4]

To date, many companies have moved this assessment to earlier stages of drug discovery in an effort to reduce costly clinical development attrition due to unfavorable safety profiles. Developing a reliable and reasonably high-throughput assay has become very critical in order to assess large numbers of drug candidates in the early stages of drug discovery. The fluoresence (FL)-based CYP inhibition assay has been widely used because it is high-throughput.[5] The luminescense-based method[6] has also received some attention recently due to its high-throughput format. However, these assays have been criticized for not using drug-like probe substrates.[3] The LC/MS-based assay utilizes conventional drug probes that are approved medicinal agents and that are CYP-enzyme specific; therefore, more and more industrial laboratories are increasingly switching to this method.[7,8]

Probe substrates must be selective, specific, and sensitive toward the CYP of interest. Over the years, pharmaceutical scientists have used numerous probe reactions for the various P450 enzymes to evaluate the inhibitory potential of a new drug. The ideal probe substrate is one which is universally accepted in the literature, widely used by the pharmaceutical industry, commercially available, and accepted by the FDA.[9] In addition, the probe substrate should provide good *in vitro* to *in vivo* correlation.[2,3,9–12] Following these criteria, six probe substrates toward five isozymes were selected for our study as follows: testosterone (TS) and midazolam (MD) for CYP3A4, and phenacetin, diclofenac, S-mephenytoin, and dextromethorphan for CYP1A2, CYP2C9, CYP2C19, and CYP2D6, respectively.

In the typical assay format, the production of metabolite following oxidation of the probe substrate is monitored by high-performance liquid chromatography (HPLC) with tandem mass spectrometry detection (LC/MS/MS). A reduction in the LC/MS/MS signal of the metabolite following the addition of a test compound indicates inhibition of CYP activity. Since chromatographic separation is needed for the LC/MS-based assay, throughput can be an issue. Many labs[13] and companies[14] have adapted a cassette-incubation technique to increase throughput of the LC/MS-based assay; however, based on our experience, cassette-incubation introduced additional DDIs for some compounds, thus, generating misleading results. Another approach is to pool samples from individual incubations for each CYP prior to LC/MS injections. Sample pooling can minimize the LC/MS time dramatically, as only one injection will be needed rather than six; however, we found that this method had the potential of compromising the MS/MS detection of strong inhibitors because each metabolite was diluted six times when combining six different incubations. The goal for this study was to develop and validate a fast and robust LC/MS-based CYP inhibition assay without such drawbacks and with relatively rapid turn-around time for early screening projects.

In this report, we present a one-point method that we developed to estimate the drug concentration at which the metabolism of the CYP probe substrate was reduced by 50% (IC_{50}). The inhibitory effects of test compounds were evaluated using human liver microsomes (HLM) and conventional probe substrates for the five CYP isoforms. Assays were conducted at one concentration (i.e., 10 μM) in duplicate. A concentration of 10 μM was chosen for the one-point assay, because it is the industry standard for initial CYP screening. Usually, it is simply reported as "%inhibition at 10 μM." Compounds with greater than 50% inhibition at 10 μM are considered "potential inhibitors," although the exact interpretation of an *in vitro* assay result at this early stage of drug development is not possible without consideration of typical concomitant medications and knowledge of efficacious drug concentrations in the patient population. We show in this paper how the one-point determination at 10 μM can be used to estimate IC_{50} values, which provides additional information to discovery-stage project teams and provides a basis for interpretation of CYP inhibition data in terminology that biologists and pharmacologists can understand. The methodology for the one-point assay were validated by correlating the results with IC_{50} values that were obtained from a traditional eight-point concentration–response curve. Throughput was increased