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Utility of *in vitro* clearance in primary hepatocyte model for prediction of *in vivo* hepatic clearance of psychopharmacons

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ABSTRACT

Primary hepatocytes offer a simple *in vitro* model for studying biotransformation of drugs or novel chemical entities. The utility of hepatocytes for the prediction of *in vivo* clearance was investigated by using 16 psychopharmacons of disparate structures (aripiprazole, biperiden, carbamazepine, citalopram, clonazepam, clozapine, duloxetine, fluoxetine, haloperidol, mianserin, mirtazapine, olanzapine, paroxetine, quetiapine, risperidone, venlafaxine). *In vitro* pharmacokinetic parameters ($t_{1/2}$ elimination half-life, Cl_{int} intrinsic clearance) were determined in human, rat, dog and rabbit hepatocytes by liquid chromatography tandem mass spectrometry, and hepatic clearance values (Cl_H), hepatic extraction ratios as well as bioavailability values were predicted. In human hepatocytes, the most stable compounds were carbamazepine, citalopram, clonazepam, fluoxetine, mirtazapine and paroxetine, displaying *in vitro* $t_{1/2}$ longer than the 5-hour incubation period, whereas quetiapine appeared to be the most labile drug. The fastest elimination rates were observed in rabbit hepatocytes, with approximately one or two magnitude orders faster than in human liver cells. *In vitro* pharmacokinetic parameters obtained from rat hepatocytes displayed strong correlation with *in vitro* human values ($r^2 > 0.85$); whereas neither the elimination rates nor Cl_{int} of the drugs in dog or rabbit hepatocytes resembled those parameters in human cells ($r^2 < 0.2$). Similarly, there were significant interspecies differences in hepatic clearance predicted from *in vitro* elimination half-lives. Namely significant correlation was observed in predicted Cl_H values between human and rat, and no correlation was found between human and dog or rabbit. The human hepatic extraction ratios of the psychopharmacons ranged widely from the lowest values for carbamazepine and clonazepam (<0.1) to the highest for quetiapine (0.7). The human bioavailability values predicted from *in vitro* pharmacokinetic data were in good agreement with clinical bioavailability data.
In conclusion, the predicted bioavailability obtained from human hepatocytes showed an excellent rank order with *in vivo* findings. Furthermore, rat was considered to be the most relevant animal model to human subjects.

1. Introduction

The early knowledge of the pharmacokinetic behaviour and the routes by which a novel drug-candidate is metabolized is important for the interpretation of toxicological data obtained in safety studies and for prediction of *in vivo* systemic clearance in human [1,2]. The hepatic metabolic clearance is considered to be the major component of the total clearance [3]; thus, in the early phase of drug development, hepatic stability screening is a widely used method to assess the metabolic stability and to predict *in vivo* hepatic clearance of a drug candidate [1,4]. For *in vivo* clearance prediction, theoretical aspects of *in vitro – in vivo* scaling have been developed and successfully established in laboratory animals or in human [5–9]. The first attempt by Rane et al. [10] calculated *in vivo* clearance from intrinsic clearance of a drug which was obtained by determination of the enzyme kinetic constants (v_{max}: maximal velocity of enzyme activity, K_M: Michaelis constant) in hepatic microsomal metabolism. The *in vitro* $t_{1/2}$ method described by Obach [6] is based on determination of the first-order rate constant for consumption of the drug-substrate for the estimation of intrinsic clearance.

The potential advantages of *in vitro* pharmacokinetic approach include the rapid distribution of the test compound, rapid sampling for kinetic studies, relatively high purity of the biological samples and the easy procedure of the determination of depletion of the parent compounds. An appropriate *in vitro* model should resemble the *in vivo*

Abbreviations: CYP, cytochrome P450; LC-MS/MS, liquid chromatography tandem mass spectrometry.

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metabolism; thus for successful prediction of *in vivo* clearance, the selection of *in vitro* models must consider the qualitative information on enzyme(s) responsible for the metabolism of a drug [1,6,7,11–15]. Several *in vitro* models have been developed, such as supersomes (expressed enzymes), subcellular fractions (microsomes, cytosol or S9 fraction), primary hepatocytes (freshly isolated or cryopreserved) or intact perfused liver, and some of these models are acceptable as supportive test systems by the regulatory authorities. Hepatic microsomes are simple, affordable and can be a useful *in vitro* model for evaluating pharmacokinetics of drugs metabolized by cytochrome P450 (CYP) enzymes [5,6]; however, this model has some drawback in studying those compounds that are metabolized by non-microsomal enzymes or by microsomal UDP-glucuronyltransferases. Conjugation activities in microsomal preparations generally underpredict *in vivo* metabolism because of the lack of conjugation enzymes (non-microsomal localisation) or insufficient activation of UDP-glucuronyltransferase activities [16]. Several authors [5,12] successfully applied primary hepatocytes for *in vitro* studies and demonstrated better predictability of *in vivo* clearance. The advantages of hepatocytes over subcellular fractions have been documented; namely the cells retain most of the metabolic capabilities of the intact liver, possess the full complement of drug-metabolizing enzymes and contain the cofactors at physiological concentrations [5,7,12,13]. Additional advantages of hepatocytes are the possibility of determination of metabolic profiles or even of metabolite identification. Hepatocytes in suspension are appropriate for the investigation of pharmacons with short elimination half-lives (<5 h), whereas liver cells attached on collagen surface are generally applied for drugs with long half-lives (>5 h). In high-throughput studies, the multi-drug-in-one-cocktail approach can be an option; however, the metabolism-based drug-drug interactions must be excluded for appropriate evaluation. *In vitro* pharmacokinetic data obtained from human hepatocytes can be used for prediction of *in vivo* hepatic clearance in human, whereas *in vitro* clearance data from hepatocytes isolated from rat, mouse, dog or rabbit can be useful to identify the laboratory animal(s) with similar pharmacokinetics to human beings. Furthermore, information on species differences in the rates and pathways of metabolism is of great interest and can help to select the laboratory animal model most relevant to human and most suitable to study the toxic properties of drugs.

In the present study, we investigated *in vitro* pharmacokinetics of sixteen psychopharmacons with disparate chemical structures (aripiprazole, biperiden, carbamazepine, citalopram, clonazepam, clozapine, duloxetine, fluoxetine, haloperidol, mianserin, mirtazapine, olanzapine, paroxetine, quetiapine, risperidone, venlafaxine, Fig. 1) in primary hepatocytes of rat, rabbit, dog and human using liquid chromatography tandem mass spectrometric (LC-MS/MS) analysis. These antipsychotics, antidepressants, mood stabilizers and anticonvulsive agents are most frequently used in treatment of psychiatric disorders (*e.g.* schizophrenia, schizoaffective disorder, bipolar disorder, depressive disorder). *In vivo* pharmacokinetic parameters (hepatic clearance, hepatic extraction ratio, bioavailability) were calculated from *in vitro* clearance data using *in vitro* $t_{1/2}$ method. *In vivo* bioavailability of the psychopharmacons predicted from human hepatocytes was compared to *in vivo* clinical data.

2. Materials and methods

2.1. Chemicals

Biperiden, carbamazepine, citalopram, clozapine, fluoxetine, mianserin, mirtazapine, paroxetine, risperidone and venlafaxine were purchased from Sigma-Aldrich Co. (Deisenhofen, Germany). Aripiprazole and duloxetine were obtained from International Laboratory USA (San Francisco, Ca). Olanzapine and quetiapine was from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Clonazepam was obtained from Roche Magyarország Ltd. (Budaörs, Hungary) and haloperidol was from Gedeon Richter Plc. (Budapest, Hungary). All the other chemicals for the hepatocyte isolation and for the LC-MS/MS analysis were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich Chemie.

2.2. Hepatocyte isolation

Experiments were carried out by using pooled hepatocytes prepared from male Wistar rats, male Beagle dogs or male New-Zealand rabbits (Toxi-Coop Toxicological Research Center, Budapest, Hungary). Human liver tissues were obtained from organ-transplant donors at the Department of Transplantation and Surgery, Semmelweis University (Budapest, Hungary). The liver cells were isolated using collagenase perfusion method of Bayliss and Skett [17]. Briefly: The liver tissues were perfused through a derivation of the portal vein with Ca^{2+}-free medium (Earle's balanced salt solution) containing EGTA (0.5 mM) and then with the same medium without EGTA, finally with the perfusate containing collagenase (Type IV, 0.25 mg/ml) and Ca^{2+} at physiological concentration (2 mM). The perfusion was carried out at pH 7.4 and at 37 °C. Hepatocytes having a viability of better than 90%, as determined by trypan blue exclusion [18], were used in the experiments.

2.3. In vitro pharmacokinetics in primary hepatocytes

Time courses of the unchanged psychopharmacons in hepatocytes pooled from three subjects were obtained. Each compound was incubated in cell suspension (generally at the concentration of 2×10^6 cells/ml; and at the concentration of 0.5×10^6 cells/ml or 4×10^6 cells/ml with the drugs intensively or slowly metabolized, respectively) at 37 °C in a humid atmosphere containing 5% CO_2. The parent compounds were added directly to the medium (Williams' medium E: Ham's nutrient mixture F12 = 1:1) at the final concentration of 1 μM. Except for clonazepam and haloperidol that were dissolved in medium, the stock solutions of the psychopharmacons (1 mM) were prepared in acetonitrile; thus, the final concentration of the organic solvent was 0.1% of the incubation mixture. At various time points (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, 300 min), the incubation mixtures were sampled (aliquots: 0.25 ml) and terminated by the addition of 0.17 ml ice-cold acetonitrile containing the internal standard, carbamazepine (0.13 μM) or clozapine (0.09 μM). The cell debris was separated by centrifugation and the supernatant was analysed by LC-MS/MS for quantitation of the parent compound. All measurements were performed in duplicate with <5% interday and intraday precision.

2.4. Determination of unchanged drug concentrations

An Inertsil ODS-4 column (75 × 2.1 mm, 3 μm, GL Sciences Inc., Tokyo, Japan) with gradient elution was applied with MilliQ water containing 0.1% of formic acid as the mobile phase A and with acetonitrile containing 0.1% of formic acid as the mobile phase B. The column was eluted at a rate of 0.3 ml/min at 25 °C using an Agilent 1100 HPLC-system (Agilent Technologies Inc., Waldbronn, Germany). The running time of chromatographic separation was 10 min, and the effluent was analysed by mass spectrometry. MS/MS measurements were performed on a SCIEX API 2000 mass spectrometer (Applied Biosystems, Foster City, Canada) using the Analyst 1.4.2 software. The ionisation mode was electrospray in positive mode. The instrument was used in multiple reaction monitoring (MRM) mode for quantitation of the psychopharmacons. The source conditions were: curtain gas: 30 units, spray voltage: 5500 V, source temperature: 400 °C, nebulising gas: 50 units, drying gas: 50 units. The MRM transitions and collision energy applied for monitoring the parent compounds are in Table 1. The primary stock solutions of psychopharmacons for the preparation of calibrator and quality control samples were prepared in acetonitrile or in water by separate weighing. The working solutions used for spiking the calibrator and quality control samples were diluted separately in acetonitrile by spiking an appropriate volume of the stock solutions to

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