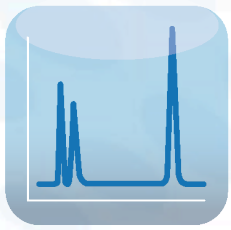
APHAD S.r.l. 2016
Catalogue

Analytical services

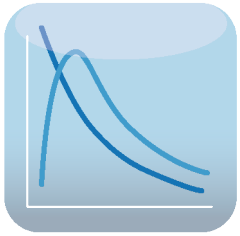


We provide to our Clients a variety of customized analyses including the classical analytical method development, impurities structure elucidation, etc.

Formulation services

Research and development of new formulations of small and large molecules, especially liquids and semi-solids, for topical, oral, nasal or other routes of administrations.
The process includes solubility studies, preliminary stability and analytical method development. A variety of solubilization methods are available. Accelerated and long term preliminary and ICH compliant stability studies can be performed.

In vitro ADMET and *in vivo* PK services

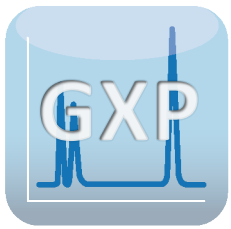


High quality ADMET data are essential to identify and help addressing rapidly the critical issues in the drug discovery, lead validation and optimization processes. A basic battery of assays is presently available. These assays can be cost effectively tailored on the Clients' needs. If a particular assay is not present in this catalogue do not hesitate to enquire. New assays are continuously developed.

While *In vitro* ADMET provides valuable indications of a compound behavior, *In vivo* studies remain essential to understand target exposure that can be achieved. APHAD therefore offers a flexible selection of procedures for the *in vivo* evaluation of compounds:

- Rodent *in vivo* PK after different routes of administration, with tissue sampling, metabolites identification and biodistribution;
- Preliminary acute and sub-chronic toxicology in rodents;
- Pharmacokinetic data analysis and report writing.

Certifications



Aphad is qualified by the Regional Authority ASL Milano 1 (Prot. N. 51058, 23/06/2014), to perform Pharmacokinetic, Metabolism and Bioequivalence, bioanalyses for clinical trials (GCP) according to art 1 and 2 of D.M. 19 March 1998. AIFA (Italian Medicines Agency) has recorded Aphad in the OsSC. Aphad was successfully inspected by the Italian Ministry of Health for preclinical GLP bioanalyses and veterinary studies on residuals. Aphad is eligible to carry out tests in compliance with GLP for 1) Physical-chemical testing 6) Residues studies and 9.7) Pharmacokinetics/toxicokinetics and ADME studies (Certificate: 2014/29)

Index of ADMET/PK Services

Artificial Membrane Permeability - PAMPA

Background: Rapid screening of passive permeability is evaluated in HTS mode using artificial membranes (PAMPA), mimicking permeability through the GI or BB barriers.

Substrate layer: Phosphatidylcoline (PC, 2%) or Polar Brain Lipids (PBL, 2%) in dodecane/hexane solution.

Test Concentration: 500 µM at two pHs, i.e. 4.0 and 7.4 (different buffers available) for intestinal permeability (PC), or at pH 7.4 for brain permeability (PBL).

Control Standards: Low, medium and high permeability compounds are added in every experiment (e.g. propranolol, cimetidine and caffeine).

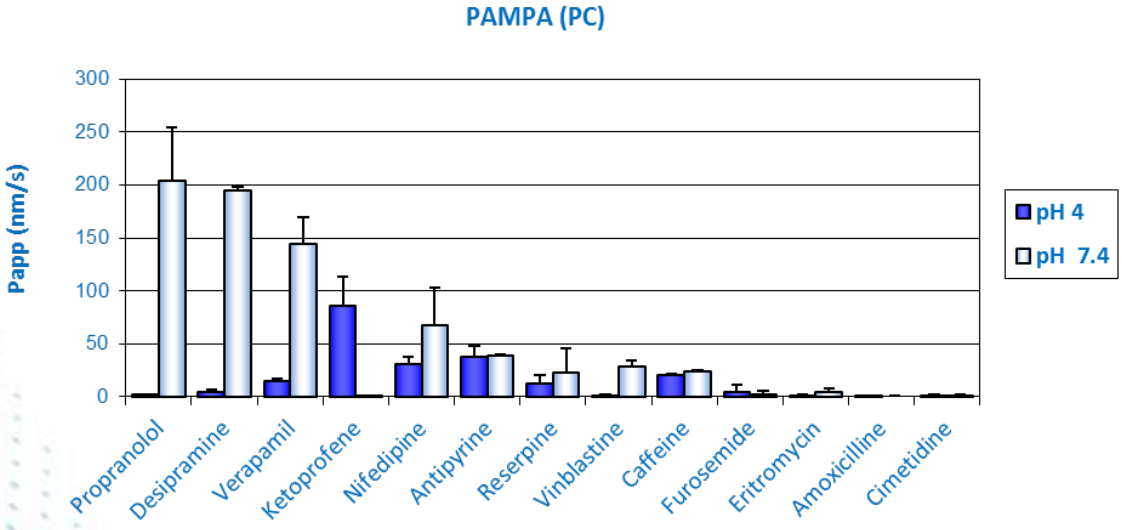
General Protocol: Permeability across the artificial layer is determined by adding the test compound solutions to the donor plate (96-well plate with porous filters pre-treated with the phospholipid solution), then assembling the donor plate with the acceptor plate containing the same buffer. After 2 hours of incubation with gentle shaking (200 rpm), the two solutions are analysed and quantified by LC-MS. Equilibrium solutions without the membrane layer are also prepared.

Analytical Method: LC-MS or MS/MS without calibration curve.

Data Analysis: Apparent permeability (Pe or Papp) is calculated according to the following equation:

$$P_{app} = \left\{(-C)*1n\left(1-\frac{[drug]_{acceptor}}{[drug]_{equilibrium}}\right)\right\}*10^{7}$$

where:
C = VD*VA/(VD+VA)*t*A
VD = volume donor solution
VA = volume acceptor solution
A = filter area
t = incubation time



Reference: Carrara S. et al., *Int. J. Pharmaceutics*, 345/1-2, 125-133, 2007.

Solubility

Thermodynamic Solubility

Background: The shake flask method is used as a reliable assay for absolute solubility determinations in different solvents/buffers.

General protocol: A calibration curve is prepared from DMSO stock solution (50 mM).
A small amount of solid compound to be tested is placed in 1 mL of the appropriate solvent and then shaken at r.t. for 24 hours. The resulting solution is filtered on a multiscreen plate then the filtrate analyzed by LC/MS or MS/MS.

Analytical method: HPLC/UV or MS/MS.

Data analysis: Solubility data from two different samples are interpolated from the calibration curve.

HTS Solubility

Background: Optimal solubility profiles are crucial in drug development. HTS methods are robust, and allow a rapid ranking of solubility among early hits.

Test Concentration: 200 or 500 µM (0.4-1% DMSO) at different pHs. Lower concentrations can be used for poorly soluble compounds.

General Protocol: 500 µM and/or 200 µM solutions in MeOH and PBS, pH 7.4 (or other buffers at different pHs) are prepared by dilution from a 50 mM DMSO stock solution for each product to be tested.
Samples are placed in a 96-well filter plate and incubated at room temperature for 90 minutes. The plate is then filtered, and solutions are analyzed by LC/MS or UV. Samples are ranked by solubility relative to reference standards used in every experiment (see Table below).

Analytical Method: HPLC/UV or HPLC/MS, without calibration curve.

Data Analysis: Final concentrations are evaluated by comparing the AUC of stock solutions in organic solvent (200 or 500µM) with those of the filtered test compound solutions.

	solubility @ pH 7.4		solubility @ pH 4.0	
Standards	**200 µM**	**500 µM**	**200 µM**	**500 µM**
diclofenac	>200	>500	<<200	<<500
phenytoin	<200	<200	<200	<200
ketoconazole	<200	<200	>200	>500

Caco-2 Permeability

Background: The intestinal permeability of compounds is evaluated in the human Caco-2 model, (three days culture) in which the flux of the test compound from the apical (A) to the basolateral (B) side (together with B to A for the FOCUS assay) is measured in order to predict the absorption from the lumen of the gut. The interaction with the active transporter P-glycoprotein (P-gp) can also be investigated.

Cells: Caco-2 monolayers cultured on cell culture inserts; transepithelial electrical resistance (TEER) >1000Ωcm2, as evidence of monolayer integrity.

Test Concentration: 10-50 µM.

Control Standards: Added in every experiment: caffeine (for high permeability), cimetidine (for low permeability P-gp substrates).

General Protocol

The transport across the Caco-2 monolayer
Can be determined (A→B) and (B→A) by adding the test compound to the apical side and the basolateral side. After 2 hours of incubation, the basolateral side solution, together with the apical and the starting solutions, are analyzed and quantified by LC-MS/MS. To maintain physiologically relevant conditions, the pH of the apical side is 6.5, while the pH of the basolateral side is 7.4. P-gp inhibitors, such as cyclosporine A, can be added to investigate efflux phenomena.
At the end of each experiment, the monolayer integrity is assessed with a lucifer yellow assay.

Analytical Method: HPLC-MS/MS (MRM), without calibration curve.

Data Analysis: The apparent permeability (Papp) is calculated according to the following equation:

$$Papp = J/Co$$

where:
J=flux (dX/dt x A) and
Co=donor concentration (µM) at
t=0; dX/dt=change in mass (X, nmol)
per time (t, sec), A=filter surface area (cm2).

The apparent permeability of the test compounds, and their rank order, is always compared with two known reference compounds tested in the same experiment. Suggested absorption classification values are:

Papp (nm/s)	classification
>50	High
10-50	Medium
<50	Low

CYP450 Inhibition

Background: Hepatic P450 enzymes are responsible for drug metabolism and drug-drug interactions. Inhibition of the most important P450 isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP2B6, CYP2E1, CYP2C8 and CYP3A5) is measured in specific assays, using specific substrates that become fluorescent upon CYP metabolism.

Species/Enzymes: Human recombinant P450 isoforms (Supersomes, Gentest).

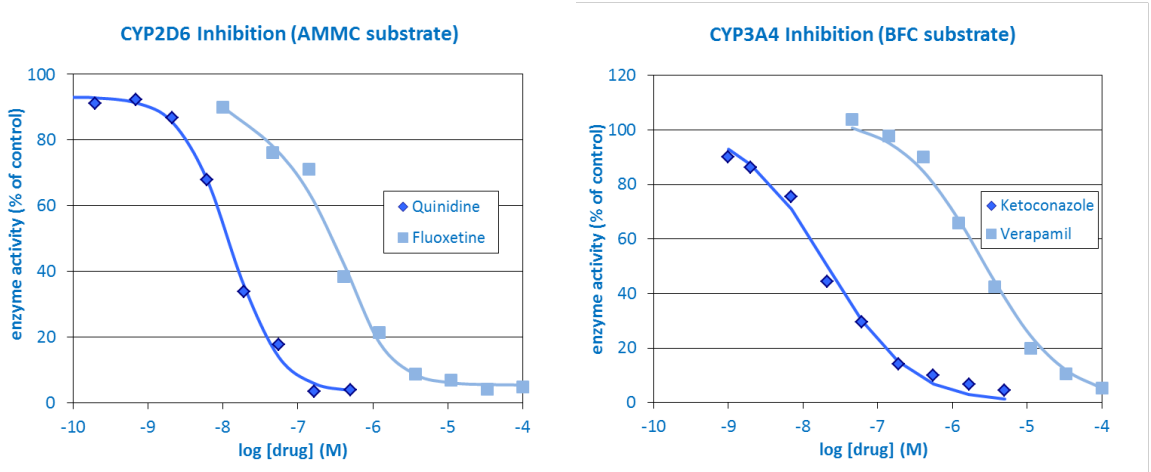
Substrates:
CEC (3-cyano-7-ethoxycoumarin) for CYP1A2 and CYP2C19;
MFC (7-methoxy-4-(trifluoromethyl)-coumarin) for CYP2C9 and CYP2E1;
AMMC (3-[2-(N,N-diethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin) for CYP2D6;
BFC (7-benzyloxy-4-(trifluoromethyl)-coumarin) for CYP3A4 and CYP3A5;
EFC (7-ethoxy-4-trifluoromethylcoumarin) for CYP2B6;
DBF (dibenzylfluorescein) for CYP2C8 and as an alternative for CYP2C9 and CYP2C19.

Test Concentration: Typically 3 µM or concentration-response curve 0.05-100 µM.

Control Inhibitors: Added in every experiment: furafylline (for CYP1A2), sulfaphenazole (for CYP2C9), tranylcypromine (for CYP2C19, CYP2B6), quinidine (for CYP2D6), ketoconazole (for CYP3A4 and CYP3A5), quercetin (for CYP2C8) and diethyldithiocarbamic acid (for CYP2E1).

General Protocol: Compound solutions are tested at single concentration or by serial dilutions in a 96-well plate containing an appropriate buffer and a NADPH regenerating system. The reaction is started by adding specific isoenzymes and substrates at 37 °C. After a 15-45 minute incubation period (depending on the CYP isoform), the reaction is stopped and plates are read on a fluorimeter at the appropriate emission/excitation wavelengths.

Data Analysis: The % inhibition of control enzyme activity or inhibition potency (IC50) is determined.



Reference: Stresser, D.M. *et al. Drug Metab. Dispos.* 28, 1440-1448, 2000.

Time-Dependent Inhibition of CYP3A4

Background: CYP enzymes are subjected to reversible, quasi-irreversible and irreversible (mechanism-based) inhibition by a number of compounds. Reversible inhibition is the result of competition at the CYP active site; as to quasi-irreversible inhibition, the metabolites may form stable complexes with the heme moiety of CYP, so that CYP is sequestered in a functionally inactive state; in the case of irreversible inhibition, the metabolites covalently bind to the CYP heme and/or protein, leading to irreversible inactivation of the CYP.

Species/Enzymes: Human recombinant CYP3A4 isoform.

Substrate: BFC (7-benzyloxy-4-(trifluoromethyl)-coumarin).

Test Concentration: Concentration-response curve, 0.05-100 µM (6 timepoints, 8 concs., n=2).

Control Inhibitors: Ketoconazole and erythromycin.

General Protocol: Compound solutions are tested by serial dilutions in a 96-well plate containing an appropriate buffer and a NADPH regenerating system. The reaction is started by adding the CYP3A4 isoenzyme and substrate at 37 °C, and fluorescence is measured by scanning the plate every 5 minutes for 30 minutes on a fluorimeter at the appropriate emission/ excitation wavelengths.

Data Analysis: The % inhibition is calculated for each time point with respect to control values (without inhibitor) at the same time point, and IC50s are calculated.

References: Naritomi, Y. *et al. Drug Metab. Pharmacokin.* 19, 55-61, 2004;
Kent, U.M. *et al. Curr. Drug Metab.* 2, 215-243, 2001.

CYP450 Phenotyping

Background: CYP phenotyping of pre-clinical candidates that are significantly unstable in presence of liver microsomes or S9 preparations, is crucial to find out which CYP isoform is involved in the Phase I oxidative metabolism.

Species/Enzymes: human recombinant P450 isoforms.

Test Concentration: 1 µM (n=2).

Control Standards: CYP1A2 (7-ethoxyresourfin, terfenadine or propranolol); CYP2C9 (diclofenac, terfenadine or propranolol); CYP2C19 (omeprazole, propranolol); CYP2D6 (dextromethorphan or propranolol, terfenadine); CYP3A4 (terfenadine, propranolol).

General Protocol:
The test compound is pre-incubated for 10 minutes at 37°C, pH 7.4 (phosphate buffer) with each CYP isoform. After the pre-incubation period, the reaction is started (time 0) by adding the cofactors mixture; samples are taken at 60 minutes or at five timepoints (0, 15, 30, 45 and 60 minutes) and added to acetonitrile containing an internal standard, to stop the reaction. Samples are then centrifuged and supernatant analyzed by LC-MS/MS. A control sample without cofactors is always added in order to check the chemical stability of the test compound.

Analytical Method: HPLC-MS/MS (MRM) without calibration curve.

Data Analysis: Compound % remaining after 60 minutes' incubation time is calculated with respect to the amount of the compound at time 0 or the rate constant k (min^{-1}), derived for the exponential decay equation, is used to calculate the rate of intrinsic clearance (Cli) of the compound using the following equation:

Cli ($\mu L \times min^{-1} \times$ mg $protein^{-1}$) = $k \times V$
where: V ($\mu L \times$ mg $protein^{-1}$) = incubation volume/mg protein added.

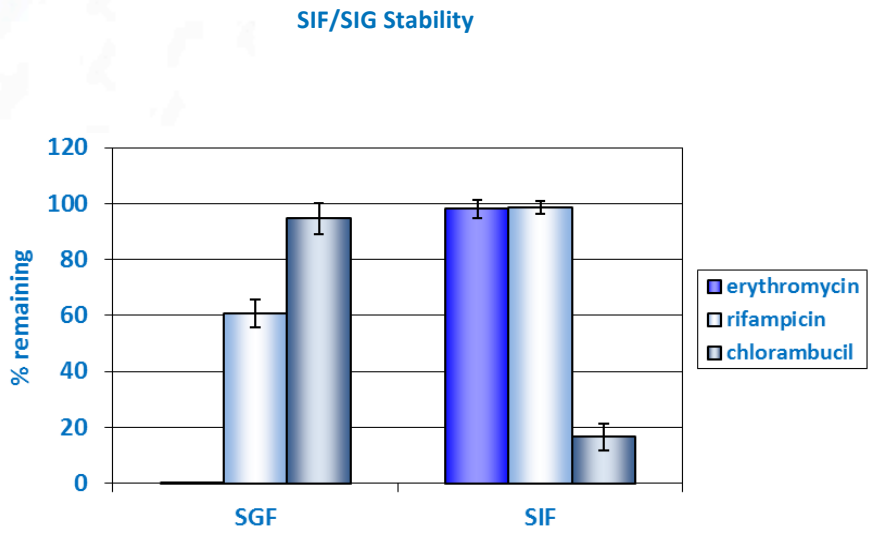
Simulated Gastric/Intestinal Fluid Stability

Background: Stability in different simulated gastrointestinal fluids (Simulated Intestinal Fluid (SIF) and Simulated Gastric Fluid (SGF)) is a key parameter to be considered, when oral administration is required. Such an assay is available for early screening of potential leads.

Test Concentration: 50 µM.

General Protocol: Kinetic studies are started by the addition of a stock solution of the compound to the incubation buffer (SGF or SIF at 37 °C, in a thermostatically controlled incubator).
The incubation media is equilibrated to the temperature of the study. At pre-determined time-points (usually between 0 and 60 minutes), aliquots are diluted with acetonitrile and analyzed.

Analytical Method: HPLC-MS/MS (MRM) or HPLC-MS. Data Analysis: The % remaining of test compound, after up to 60 minutes' incubation time, is calculated with respect to the amount of the test compound at time 0.



References: Ehrsson, H. *et al., Journal of Pharmaceutical Sciences,* 69, 1091-4, 1980;
Singh, S. *et al., Pharm. Pharmacol: Commun.,* 6, 491-4, 2000.

Metabolic Stability

Background: Hepatic enzymes are responsible for drug metabolism. The stability of the test compound can be assayed in microsomes and S9 liver subcellular fractions or hepatocytes.

Species/Tissues: Human, monkey, dog, guinea pig, rat and mouse - liver microsomes or S9 and cryopreserved hepatocytes; Other tissues (e.g. rat pulmonary microsomes) from different species may be available. Please inquire.

Test Concentration: 0.5-5 µM.

Control Standard: Added in every experiment: 7-ethoxycoumarin and/or a chemical class-related compound.

General Protocol: The test compound is pre-incubated for 10 minutes at 37 °C in the appropriate buffer at pH 7.4 with microsomes or S9. After the pre-incubation period, the reaction is started (time 0) by adding the cofactors mixture;
For testing also Phase II (i.e. glucuronidation reaction) liver microsomes are pre-incubated alamethicin prior the addition of the substrate. After 10 min at 37 °C, UDPGA (5 mM final concentration) and the cofactor mixture are added to start the reaction. Samples are taken at 30 minutes or at six timepoints (typically at 5, 10, 15, 2 and 30 min) and added to acetonitrile containing an internal standard to stop the reaction. Samples are then centrifuged and supernatant analyzed by LCMS/MS. A control sample without cofactors is always added in order to check the chemical stability of test compound in the matrix. The same protocol is followed using cryopreserved hepatocytes incubating the test compound without the cofactor mixture.

Analytical Method: HPLC-MS/MS (MRM) without calibration curve.

Data Analysis: % remaining of compound after 30 minutes of incubation time is calculated with respect to the amount of the compound at time 0 or the rate constant k (min^{-1}), derived for the exponential decay equation, is used to calculate the rate of intrinsic clearance (Cli) of the compound using the following equation:
Cli (µL × min^{-1}× mg $protein^{-1}$) = k × V
where: V (µL × mg $protein^{-1}$) = incubation volume/mg protein added.

Plasma Stability

Background: Compounds that show intrinsic instability in plasma are obviously unsuitable drug candidates. Several assay formats are available to assess the stability in plasma of test compound, using plasma from different species.

Species: Mouse, rat, dog, human, monkey and guinea-pig
(other species, please inquire).

Test Concentration: 2.5 µM.

General Protocol: Prior to the kinetic study, the incubation media are equilibrated to the temperature of the study. Kinetic studies are initiated by the addition of a stock solution of the test compound to plasma at 37 °C, yielding an initial concentration of 2.5 µM. At pre-determined time intervals or at 60 minutes, sample aliquots are removed and proteins are precipitated by addition of acetonitrile.

Analytical Method: HPLC-MS/MS without calibration curve containing an internal standard. Samples are centrifuged for 10min at 13000rpm at 15 °C and supernatant analyzed by LC-MS/MS.

Data Analysis: Compound % remaining after 60 minutes' incubation time is calculated with respect to the amount of the compound at time 0. Alternatively, the rate constant k (min^{-1}), derived for the exponential decay equation, is calculated.

Reference: Li Di *et al., Int. J. of Pharmaceutics,* 297, 110-119, 2005.

Metabolite Identification Studies

Background: A variety of enzymes present in different organs/tissues may be responsible for drug metabolism. Early investigation of metabolic pathways, detection and/or identification of potential metabolites is essential during the lead optimization phase, i.e. well before toxicological studies. Furthermore an understanding of the metabolism of a compound gives clear guidance to medicinal chemists in optimizing PK within a chemical services.

Species/Tissues: Human, monkey, dog, guinea pig, rat and mouse liver microsomes and S9 fraction; plasma and/or other biological matrices from in vivo treatments.

General Protocol: The test compound (10 µM) is incubated at 37 °C with the biological matrix of interest. After 60 to 90 minutes, the samples are added to acetonitrile to stop the reaction, and then centrifuged. The supernatant is finally analyzed by LC-$(MS)^n$ (ABSciex 4500 QTrap).

Data Reporting: The following deliverables will be supplied:

- summary of Materials and Methods
- reconstructed chromatograms of parent and potential metabolites
- mass spectra of major peaks of the chromatograms
- product ion spectra for parent and putative metabolites
- preliminary analysis of the parent compound sites that underwent metabolic attack.

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Cellular Toxicity

Background: The cytotoxic potential of test compounds is evaluated via a rapid MTT assay in the hepatoma cell line.
The MTT format refers to the cytotoxicity test based on the reduction of the soluble dye MTT (3-(4,5-dimethylthiazyl-2-yl) 2,5 diphenyltetrazolium bromide salt) to a blue insoluble formazan product by mitochondrial succinic dehydrogenase.

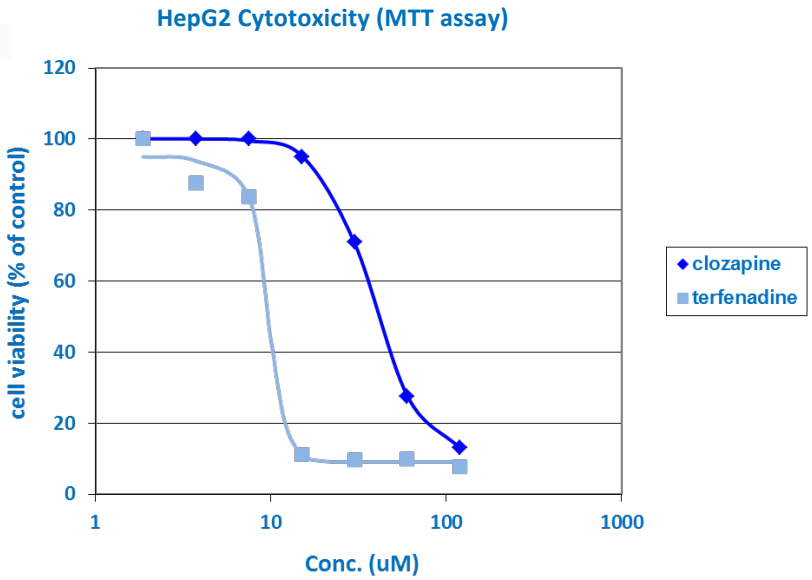
Cells/Tissues: HepG2 cells

Test Concentration: Concentration-response curves, 1-100µM

Standards: Terfenadine or clozapine (added in every experiment).

General Protocol: HepG2 cells are seeded in 96-well tissue culture plates (90 µl/well) and incubated overnight at 37 °C. The following day 10 µl/well of medium, vehicle or test compound is added to the cells, and incubated for 48 hours at 37°C. After incubation, medium is discharged and 100ul/well of the initial medium with 0,1% of FBS and 20ul/well of MTS (Promega) are added. Plate is again incubated for 4hrs and absorbance is read at 490nm.
The inhibition of fluorescence produced in respect to the control is considered as cytotoxic effect (MTS tetrazolium reduction).

Data analysis: % of cell viability after incubation with the test compound is calculated with respect to the vehicle-treated cells. Concentration-response curves are analyzed and the cytotoxic potency (IC50) is determined.



In vivo Pharmacokinetics

Background: Although *in vitro* ADMET is essential in driving medicinal chemistry strategy, efficient identification of a high quality preclinical candidate compound can only be achieved with extensive characterization of compounds' properties in rodents and/or, when needed, in other species. Pharmacokinetics studies can be performed in rodents, i.e. rats or mice, using different routes of administration - i.v., p.o., i.p., i.m., s.c.. All the experiments are performed in agreement with Italian Law (D. L.vo 116/92).

Test Compound: The client shall provide available information on the physico-chemical properties of the test compound, including specific indications on the final formulation (i.e. dose, solvent and dose volume) for the intended route of administration along with any available safety information.

Formulation Studies: If no indications on the formulation are available for the intended route of administration (i.e. solvent and dose volume), a preliminary study can be conducted testing different solvents/vehicles (e.g., pH manipulations where applicable, co-solvents, surfactants and complexing agents) suitable for the intended route of administration. No extra charge for basic pre-formulation studies will be added.

Analytical Method Development: Standard optimization of MS detection and the determination of LC conditions for suitable sensitivity and specificity are performed routinely included in the price. The most appropriate extraction method for the test compound from each biological matrix is identified through recovery evaluation. Eight point calibration curves (typically 1000-1 ng/ml, if feasible) are generally used. QC samples of the test compound at three different concentrations (high, medium and low) will be considered, with an accuracy within ±15% except at the LLOQ, where ±20% is accepted. GLP-like analytical development method can be set-up upon request (subject to a surcharge).

***In vivo* PK:** Administration of test compounds (acute treatment schedules) are performed using different routes of administration (e.g., i.v., p.o., i.p., i.m., s.c).
Chronic treatments and multiple administration schedules are also available – please inquire.

Surgical procedures: Some surgical procedures are available to conduct specific pharmacokinetic studies, e.g.:

- a polyurethane catheter, connected with a Silastic cannula, can be surgically inserted into a jugular vein of anaesthetized rats in order to perform animal treatment and/or sampling (Kurowsky S.Z. et al., J. of Pharmacol. Methods 26, 249-56, 1991);
- an Alzet osmotic pump can be surgically implanted subcutaneously for infusion of test compounds (Struyker-Boudier H.A.J. et al., J. Pharm. Pharmac. 30, 576-578, 1978).

Blood Sampling - Acute Treatment: Typically for rats (n=3) 8 time points, serial sampling from the caudal vein; for mice (n=24) 8 time points, parallel sampling (n=3, per time point). No overnight sampling is performed routinely (last time point is 8 hours, 24 hours' sampling can be performed), however different sampling schedules can be agreed if needed.

Metabolic cages: Rats or mice can be placed in metabolic cages in order to collect urine and faeces for disposition studies of test compounds. The schedule of the treatment and samples collection shall be agreed in advance with the client.

Bioanalysis - External Samples: Biological samples obtained after external treatments can be analyzed in our laboratories using our bioanalytical platform. Quantification of the test compound in tissue/organ homogenates is performed using the same criteria as described in the Analytical Method Development section.

Disposition and Distribution Studies: Different tissues and organs (e.g. brain, liver, lung and kidney) can be collected at different time points in order to study the distribution of the test compound in compartments other than plasma. The schedule of the treatment and samples collection shall be agreed in advance with the client. Quantification of the test compound in tissue/organ homogenates is performed using the same criteria as described in the Analytical Method Development section.

Metabolite identification: Deliverables and data reporting, please see page 12.

In life plasma/tissues sampling: The sampling protocol is agreed with the client for each experiment.

Reporting: A summary report of Materials and Methods including the experiment schedule and the relevant PK parameters (i.e. AUC, Cmax, Tmax, t., Vd and Cli after IV dosing, non-compartmental analysis, Phoenix 6.3.0) will be given. A GLP-like report can also be issued upon client request (subject to a surcharge).

Experience and Creativity for rent...

...Accountability included

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