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In vitro safety cardiovascular pharmacology studies: Impact of formulation preparation and analysis

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ABSTRACT

Collection of formulation samples is required for GLP *in vitro* studies to check the exposure of the test system and allow reliable determinations of safety margins. *In vitro* studies conducted in-house were investigated to evaluate problems of solubility, stability and adsorption of the formulations. Terfenadine was used as reference substance to illustrate the purpose. Lowered target concentrations of test substances in *in vitro* studies can be attributed to the solubility limitation in the superfusion medium, the low stability under frozen conditions (24% of the final solutions stable at −20 °C) and/or the adsorption on the superfusion tubing (30% of the studies). Terfenadine also showed a limited solubility (measured concentrations ranging from 0.597 μM to 0.833 μM instead of 1 μM) and a loss of substance through the superfusion tubing from −30.2% to −39.2% with dimethylsulfoxide, ethanol or methanol. Terfenadine solubility was improved with 2-hydroxypropyl-β-cyclodextrin, no adsorption was observed, but its capacity to block the hERG channel was decreased. It is recommended to determine the substance solubility in appropriate buffers, to evaluate possible adsorption during method validation (formulation samples collected after superfusion), and to prepare fresh formulation each testing day with immediate analysis in absence of stability data. This strategy clearly favors single-site as opposed to multi-site studies.

1. Introduction

The assessment of the potential for QT interval prolongation is now part of the evaluation process required by the regulatory agencies for new chemical entities (NCE). Besides *in vivo* studies (QT/QTc assay in conscious large animals using telemetry), the ICH S7B guideline recommends the evaluation of NCE in *in vitro* electrophysiological assays such as Purkinje fiber or human ether-a-go-go-related gene (hERG) channel (ICH Harmonized Tripartite Guideline (S7B), 2005). The *in vitro* hERG channel test is commonly used as an early screen to predict the ability of a drug to prolong the QT interval (Pollard et al., 2010). Indeed, most of the compounds that have been withdrawn from the market because of the occurrence of *torsade de pointes* have been associated with a direct block of hERG potassium channels or its native current, the rapidly activating delayed rectifier potassium current (I_{Kr}) (Gintant et al., 2006; Picard et al., 2011; Gintant, 2011).

In *in vitro* tests, the standard superfusion solutions used are usually aqueous physiological buffers such as Hepes-buffered salt solution or Tyrode's solution. Nevertheless, difficulties to solubilize NCE in these media and thereby to achieve the expected concentrations in the bath are often encountered. Sometimes, the limits of solubility with the substance in the superfusion medium require the use of solvents or excipients to achieve the appropriate target concentrations (Himmel, 2007). Despite the use of solvents, the final superfused solutions are not always soluble and the exposure of preparations (such as Purkinje fiber) or human embryonic kidney (HEK) 293 cells stably transfected with the hERG channel might thereby be underestimated. As a consequence, collection of formulation samples becomes a necessity for *in vitro* studies conducted in compliance with Good Laboratory Practice (GLP), to check the exposure of the test system and to allow accurate and reliable determination of safety margins. It is hence important to possess a specific and robust analytical method which should be validated for each test substance before initiating the experimental phase in order to verify the concentrations applied to the test system. Such analytical methods typically involve the interpolation of unknown superfused concentrations against a calibration curve of the test substance prepared in the same matrix and over a suitable range of concentrations (Herron et al., 2004).

The aim of the present work was to systematically and comprehensively analyze *in vitro* (hERG channel and Purkinje fiber) studies conducted in-house and to highlight issues frequently encountered in these studies with the preparation and the analysis of the superfused solutions: solubility, stability and adsorption of

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the formulations. To illustrate the purpose, terfenadine was used as reference substance.

2. Materials and methods

2.1. Formulation preparation

2.1.1. Standard preparation of test substances in in vitro studies

If soluble, the test substances were directly prepared in the superfusion solution which served as vehicle (Tyrode's solution for the Purkinje fiber studies or extracellular solution for the patch-clamp studies).

If insoluble, the test substances were dissolved by stirring in pure dimethylsulfoxide (DMSO) to provide a stock solution concentrated 1000-fold as compared with the highest concentration to be tested. This stock solution then served to prepare the solutions containing the final concentrations to be tested by dilution in extracellular solution. The final concentration of DMSO never exceeded 0.1%. Test substances were prepared in glass containers to limit the contact with the plastic material during the phase of formulation preparation.

2.1.2. Terfenadine preparation for hERG channel test

2.1.2.1. Use of dimethylsulfoxide, ethanol or methanol. Terfenadine was dissolved in pure DMSO, ethanol or methanol to provide a stock solution at 3.33 or 10 mM. The recovery obtained on the filtered stock solutions was in the specified range (90–110%). Final solutions were then obtained by dilution in extracellular solution, containing the following components (mM): K-gluconate: 4/Na-gluconate: 145/Mg-gluconate: 2/Ca-gluconate: 3.5/HEPES: 5/glucose: 5/mannitol: 20 (pH = 7.40 ± 0.05).

2.1.2.2. Use of 2-hydroxypropyl-β-cyclodextrin. Terfenadine was dissolved in 0.3% 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) solution prepared in extracellular solution to give a stock solution at 1 mM. This stock solution was maintained under magnetic stirring for at least 24 h and then filtered on a 0.45 μm filter before analysis. The recovery obtained after filtration was approximately 19% of the theoretical concentration. The final solutions (0.01, 0.1, 1 and 10 μM) were obtained by an appropriate dilution of the stock solution in the vehicle (0.3% HP-β-CD in extracellular solution) taking into account the recovery after filtration.

2.2. Formulation sampling

Appropriate samples of the formulations were taken for analyses of test substance concentrations. Formulation samples (approximately 1 ml each) were taken in the reservoir bottle (before superfusion) from each test substance solution, from the stock solutions and from the vehicle. For the final superfused solutions, the samples were also collected at the end of the superfusion tubing in order to evaluate possible adsorption. The superfusion system was made using 10-ml Terumo syringe (test substance reservoir). Each polyethylene syringe was connected via C-Flex (approximately 19 cm) and polyethylene (approximately 30 cm) catheters to a borosilicate glass capillary tube. The collected samples were then either analyzed immediately or stored under frozen conditions (−20 ± 5 °C) for stability assessment.

2.3. Formulation analysis

Quantitative determination of each test substance concentration was assayed using high performance liquid chromatography (HPLC) method with UV detection which was validated in terms of specificity, linearity, accuracy, precision and effect of dilution before starting the electrophysiological experiments. The validation experiments follow international guidelines (ICH Harmonized Tripartite Guideline (Q2R1), 2005). The analyses were performed on an Agilent chromatographic system coupled with the software EZ Chrom Elite version 3.3.2 from Agilent.

For terfenadine evaluation, the chromatographic conditions were as follows: Analytical column: Advantage Lancer C18, 150 × 3.0 mm, 5 μm, Part N°ADV5058; Column temperature: +30 °C; Sample temperature: +20 °C; Mobile phase: water/acetonitrile (50:50, v:v) + 0.1% formic acid (pH 6.0); Flow rate: 1.0 mL/min; Detector wavelength: 210 nm; Injection volume: 30 μl; Approximate Retention time: 4.8 min and Run time: 8 min.

The specificity of the method was established by the absence of signal at the retention time of terfenadine in blank sample (DMSO diluted in extracellular solution). Linear responses were observed within the calibration range (0.5 to 12.7 μM of terfenadine). Accuracy and precision (repeatability and intermediate precision) were assessed on terfenadine final solutions and the effect of dilution was checked on terfenadine stock solution in DMSO. Accuracy values ranged from 85% to 105% and relative standard deviation (RSD) was below 3%. The lower limit of quantification was 0.3 μM. Final solutions at 0.01 and 0.1 μM could not therefore be analyzed.

The results of the analyses were considered sufficient if the measured contents of the test substance were within 90–110% of the theoretical concentrations and if RSD was below 3%.

2.4. Test system

Human embryonic kidney (HEK293) cells were stably transfected by the hERG clone (Creacell, La Tronche, France) and maintained at 37 °C in a 5% CO_2/95% air incubator.

The experiments were performed in accordance with the French legislation concerning the importation and housing of genetically modified cells belonging to Class 1, Group I, Confinement L2 and in accordance with a currently valid license, issued by the French Ministry for Research.

2.5. Patch-clamp recording: hERG channel test

Cells used for the study were transferred to an experimental chamber of approximately 2 ml which was maintained at a temperature of 35 ± 0.5 °C by a thermoelectric device (Harvard Apparatus: Type TC-344B) and mounted on the platform of an inverted microscope (Olympus: Type IX-51), as previously described (Goineau et al., 2012). Cells were continuously superfused with Tyrode's solution, containing the following components (mM): NaCl: 145/KCl: 4/HEPES: 5/glucose: 5/$CaCl_2$: 1/$MgCl_2$: 1 (pH = 7.45 ± 0.05).

Ionic currents from hERG-transfected cells were measured using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Glass pipettes were pulled from borosilicate glass by a vertical puller (Sutter Instruments: Type P30). Pipette tip resistance was approximately 1.5 to 3.5 MΩ when filled with internal solution, containing the following components (mM): K-gluconate: 145/Mg-gluconate: 1/EGTA: 2/HEPES: 5/K_2ATP: 2 (pH = 7.20 ± 0.05).

The pipettes were connected to the input stage of a patch-clamp amplifier (Axon Instruments: Multiclamp 700B). Stimulation, data recording and analysis were performed using specialized Axon Instruments software (pClamp 9.2.0.).

After rupture of the cell membrane (entering whole-cell mode), cells were stimulated every 10 s (0.1 Hz) using the following protocol: 500 ms pulse to +10 mV from a holding potential of −80 mV followed by a 500 ms pulse to −40 mV during which tail current was measured. The cells were considered as valid if the following criteria were achieved: cell capacitance <80 pF, access resistance <20 MΩ and holding current >−200 pA.