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Development and validation of a method for the analysis of hydroxyzine hydrochloride in extracellular solution used in in vitro preclinical safety studies

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ABSTRACT

In the process of drug development, preclinical safety studies are to be performed that require the analysis of the compound at very low concentrations with high demands on the performance of the analytical methods. In the current study, a UPLC–MS/MS method was developed and validated to quantify hydroxyzine hydrochloride in an extracellular solution used in a hERG assay in concentrations ranging from 0.01 to 10 μM (4.5 ng/ml–4.5 μg/ml). Chromatographic separation was achieved isocratically on an Acquity BEH C_{18} analytical column. The assay was validated at concentrations of 0.11–1.1 ng/ml in end solution for hydroxyzine hydrochloride. Linearity was demonstrated over the range of concentrations of 0.06–0.17 ng/ml and over the range of concentrations of 0.6–1.7 ng/ml in end solution with the coefficient of correlation $r > 0.99$. Accuracy of the achieved concentration, intra-run, and inter-run precision of the method were well within the acceptance criteria (being mean recovery of 80–120% and relative standard deviation ≤10.0%). The limit of quantification in extracellular solution was 0.09 ng/ml. Hydroxyzine hydrochloride in extracellular solution proved to be stable when stored in the fridge at 4–8 °C for at least 37 days, at room temperature for at least 16 days and at +35 °C for at least 16 days. The analytical method was successfully applied in hERG assay.

1. Introduction

Hydroxyzine, a first generation H_1 receptor antagonist, is still widely used in generalized anxiety disorder [1]. Regulatory guidelines ICH S7b require investigation of the liability for delayed ventricular repolarization. Therefore, preclinical safety pharmacology studies such as the Human ether-a-go-go-related gene (hERG) assay are performed before the first human dosing [2]. However, hydroxyzine the active ingredient of Atarax™ was launched in 1955. At that time, the hERG study was not yet required. Since 2000, different values of IC50 for hERG K^+ current inhibition were published for hydroxyzine ranging from 0.16 to 10 μM [3–5]. To clarify and better understand the discrepancies between these published values, UCB Pharma S.A. conducted its own hERG study under Good Laboratory Practice conditions. Therefore, a robust analytical method was required to check the hydroxyzine concentration in extracellular solution. Several analytical methods were developed however none of them were suitable for the analysis of hydroxyzine hydrochloride in extracellular solution due to a lack of sensitivity, precision, and/or accuracy [6–11]. The analytical method development was challenging because very low concentrations of hydroxyzine in the presence of high salt concentrations have to be quantified requiring an accurate and precise analytical method. In the present study, the analytical development, the method validation and the formulation sample analysis are summarized.

2. Materials and methods

2.1. Chemicals and materials

Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, glucose, and sodium hydroxide were purchased from Merck (Darmstadt, Germany) and 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) was purchased from Invitrogen Gibco (Auckland, New Zealand).

The hydroxyzine hydrochloride (molecular weight = 447.83 g/mol) and 2-(2-{2-[4-(diphenylmethylidene)

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Table 1
Mass spectrometer parameters.

Analytes	Parent ions (*m*/*z*)	Daughter ions (*m*/*z*)	Collision energy (eV)	Cone voltage (V)	Capillary voltage (kV)	Cone gas flow (l/h)	Nitrogen collision gas flow (ml/min)	Nebulizer gas pressure (bar)
Hydroxyzine	375.2	201.1	18	40	0.9	150	0.15	7
Internal standard (IS)	382.2	276.2	24	60	0.9	150	0.15	7

piperidin-1-yl]ethoxy}ethoxy) ethanol (Internal Standard; molecular weight = 417.98 g/mol) were obtained from process chemistry at UCB Pharma S.A. (Braine L'Alleud, Belgium)

Ammonium acetate, acetic acid, acetonitrile, and methanol were purchased from Biosolve (Valkenswaard, The Netherlands).

Acquity UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm particle size) was purchased from Waters (Milford, MA, USA) and reagent-grade water was obtained using a Milli-Q water purification system from Millipore (Bedford, MA, USA)

2.2. Equipment

The UPLC–MSMS system included an Acquity Ultra Performance LC and a Xevo TQS triple-quadrupole mass spectrometer (Waters,Milford, MA, USA).

2.3. Preparation of standard solutions and samples

The extracellular solution was a mixture of 137 mM sodium chloride (NaCl), 4 mM potassium chloride (KCl), 1.8 mM calcium chloride ($CaCl_2$), 1 mM magnesium chloride ($MgCl_2$), 10 mM glucose and 10 mM HEPES in Milli-Q water. The final pH of the extracellular solution was adjusted to 7.4 with NaOH 1N.

Stock solution was prepared at a concentration of 1 mM by weighing 22.4 mg of the test substance into a 50 ml volumetric flask and filling with extracellular solution. 0.01, 0.1, 1, and 10 μM standard solutions were made up from a stock solution and/or dilutions of this solution.

Internal standard solution was prepared at a concentration of 62 μg/l by weighing 6.2 mg of the internal standard substance, making up to a volume of 1000 ml with mobile phase and diluting by a factor of 100 with mobile phase.

From the 1 and 10 μM solutions, five samples were taken and diluted with extracellular solution to a concentration of 0.1 μM. From each dilution, 250 μl was pipetted into a 10 ml volumetric flask (i.e., 40-fold dilution). After adding 100 μl internal standard solution, the flask was filled to the mark with mobile phase to obtain the end solution ready for injection.

From the 0.01 μM and 0.1 μM solutions, five samples of 250 μl of each concentration were pipetted into 10 ml volumetric flasks (i.e., 40-fold dilution). After adding 100 μl internal standard solution, the flasks were filled to the mark with mobile phase to obtain the end solution ready for injection.

Calibration solutions in the concentration range of 0.005–0.015 μM and 0.05–0.15 μM were prepared in extracellular solution and were diluted by a factor of 40 according to the procedure above prior to injection.

2.4. Chromatographic conditions

Chromatographic separation was performed on an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle size). An isocratic elution program was used with aqueous phase/methanol/acetonitrile (45:36:19, v/v/v), aqueous phase was 10 mM ammonium acetate adjusted to pH 4.0 with acetic acid. The flow rate was 0.3 ml/min and the injection volume was 10 μl (full loop). The column temperature was maintained at 40 °C.

Table 2
Method validation acceptance criteria.

Parameter	Acceptance criteria
Specificity, selectivity and carryover	S/N <10 for interfering peaks
Sensitivity (LOQ)	S/N≥10, accuracy 80–120% and RSD≤10%
Linearity	r > 0.99 and back calculated concentrations within 20% deviation of the nominal values
Accuracy	80.0–120.0% of the expected content
Repeatability	RSD≤10.0%
Within laborattory reproducibility	RSD≤10.0%

2.5. Mass spectrometry conditions

The target compound (hydroxyzine) and the internal standard (2-(2-{2-[4-(diphenylmethylidene) piperidin-1-yl]ethoxy}ethoxy) ethanol) were detected by electrospray spectrometry (ESI/MS/MS) using multiple reaction monitoring (MRM) in positive ionization mode. Determination was performed using a Waters Xevo TQS triple-quadrupole mass spectrometer equipped with an ESI source. The parameters of the mass spectrometer under the ESI+ mode are summarized in Table 1.

2.6. Method validation

The analytical method was validated according to the International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH) [12]. The acceptance criteria are summarized in Table 2. In addition solute stability was evaluated under various storage conditions.

2.7. Dummy run analysis

The dummy run is done to check any adsorption of the analyte in extracellular solution onto glass or plastic in the perfusion system of the hERG assay [13]. The perfusion system (Fig. 1) is made up of different materials such as polytetrafuloroethylene, polypropylene, and other trade mark materials. The lack of adsorption was demonstrated by calculating the percent change from the analyte concentration in the solution reservoir to the analyte concentration after perfusion through the delivery system. Five analyte concentrations ranging from 0.01 to 3 μM were perfused in the system at two distinct temperatures (room temperature and 35 °C). Samples were collected before and after perfusion and from the wash out solution after 6 and 11 min.

2.8. Formulation sample analysis

The purpose of formulation analysis is to verify that the in-vitro test system (in this case, mammalian cells expressing human hERG