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Physicochemical characterization of asulacrine towards the development of an anticancer liposomal formulation via active drug loading: Stability, solubility, lipophilicity and ionization

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ABSTRACT

To facilitate the development of a liposomal formulation for cancer therapy, the physicochemical properties of asulacrine (ASL), an anticancer drug candidate, were characterized. Nano-liposomes were prepared by thin-film hydration in conjugation with active drug loading using ammonium sulphate and post-insertion with Poloxamer 188. A stability-indicating HPLC assay with diode array detection was developed for the determination of ASL concentrations. The U-shaped pH-solubility profile in aqueous solutions, with a lowest solubility at pH 7.4 (0.843 μg/mL), indicated that ASL is an ampholyte, and dilution or neutralization of acidic drug solutions used in clinical trials with physiological fluids may cause drug precipitation. The basic pK_a value measured by absorbance spectroscopy was 6.72. The log *D* value at pH 3.8 was 1.15 which increased to 3.24 as pH increased to 7.4. ASL was found to be the most stable in acidic conditions and degraded most rapidly in alkaline conditions. An extra-liposomal pH of 5.6 during drug loading was found to be optimal to achieve the highest drug loading (DL) of 4.76% and entrapment efficiency (EE) of 99.9%. At this pH, >90% of ASL was ionized conferring high drug solubility (1 mg/mL) and acted as a reservoir of unionized ASL to be transported into liposomal cores. As a suspension the optimized liposomes showed great physicochemical stability for five months at 4 °C. In summary, the obtained physicochemical parameters provided insightful information useful to maximise DL into the liposomes, and explain a tendency of drug precipitation of pH-solubilized formulations following intravenous infusion.

1. Introduction

Asulacrine (ASL, 9-[(2-methoxy-4-methylsulphonylamino) phenylamino]-*N*,5-dimethyl-4-acridinecarboxamide), is an inhibitor of topoisomerase II (Schneider et al., 1988). ASL was developed at the Auckland Cancer Society Research Centre, The University of Auckland, and is an analogue of the anti-leukaemia drug amsacrine (Baguley et al., 1984; Cain et al., 1975), but has a broader anti-tumour spectrum than amsacrine including activities against solid tumours such as breast and lung cancer (Baguley et al., 1984; Sklarin et al., 1992). Its anti-tumour action is mediated through DNA breakage and formation of DNA protein cross-links that lead to cell death (Baguley, 1984, 1990; Covey et al., 1988).

ASL advanced to phase I/II clinical trials where it was administered by intravenous (i.v.) infusion (Fyfe et al., 2001; Hardy et al., 1988; Sklarin et al., 1992). Although ASL showed promising clinical data in antitumour activity, the high incidence of phlebitis (53%) caused by i.v. infusion of ASL solution (1 mg/mL, using isethionate salt) was associated with pain, erythema and swelling of the arm (Fyfe et al., 2001; Sklarin et al., 1992). Further progress of clinical trials is not possible unless new formulations are developed to reduce administration associated side effects (Sklarin et al., 1992).

Intravenous infusion of many anticancer drugs, including bisantrene, amsacrine, doxorubicin, epirubicin, 5-fluorouracil, is associated with phlebitis – inflammation of the veins (Cassileth and Gale, 1986; Powis and Kovach, 1983; Suga et al., 2009; Yamada

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et al., 2011). The mechanisms by which i.v. infusion causes phlebitis have not been well defined; drug irritancy and/or post-injection drug precipitation (PIP) have been associated (Powis and Kovach, 1983; Yalkowsky et al., 1983, 1998). PIP is problematical resulting in low and variable systemic bioavailability (Tuttle, 1977; Wu et al., 2013), and tissue damage occurs due to long-time exposure of the drug to the local tissues (Wu et al., 2010) and mechanical irritation (Yalkowsky et al., 1998; Yalkowsky et al., 1983; Yamada et al., 2011). We have found that dilution of the ASL solution used in clinical trials with a phosphate buffer (pH 7.4, 0.1 M) caused immediate drug precipitation, indicating PIP may have occurred during i.v. infusion as the pH of the formulation changes to 7.4 (Wu et al., 2005b). A new formulation for prevention of PIP is essential to avoid phlebitis during i.v. administration of ASL.

Liposomes are biocompatible vesicular systems consisting of phospholipids bilayers with aqueous cores which are capable of carrying both water soluble and lipid soluble drugs. Nano-scale liposomes are well documented to be efficient tumour-targeting delivery systems by exploiting the EPR (enhanced permeability and retention) effect (Matsumura and Maeda, 1986) and enhancing drug distribution in solid tumours (Allen et al., 1991; Drummond et al., 1999; Gubernator, 2011; Lasic, 1997; Torchilin, 2005; Voinea and Simionescu, 2002; Yuan et al., 1994). Liposomes, by separating the encapsulated drug from the surrounding tissue fluid, are expected to prevent PIP, thereby potentially reducing phlebitis (Hu et al., 2011; Liu et al., 2013).

To develop a liposomal formulation, the physicochemical properties of the drug, solubility, ionization constant (pK_a), distribution coefficients ($\log D$s) as well as chemical stability as a function of pH must be determined to facilitate the design and formulation of liposomes. Furthermore, accurate characterization of drug properties is crucial in developing high-dose i.v. infusion regimens for poorly water soluble drugs to prevent PIP (Jain et al., 2010). So far, very limited physicochemical data of ASL are reported including pK_a (pK_a 6.4) and partition coefficient ($\log P$ 1.1) (Paxton and Jurlina, 1986), however, no experimental processes are described. In addition, although HPLC methods for the detection of ASL in the presence of its metabolism products have been published (Ganta et al., 2008; Jurlina and Paxton, 1985), there is no stability-indicating HPLC assay specific for the determination of ASL in the literature. The aim of the present work was to fully characterize the physicochemical properties of ASL, including ionization, lipophilicity, solubility and stability. The obtained information was applied in preparing ASL liposomes to achieve high drug content through a remote loading method using a transmembrane gradient of ammonium sulphate. A rapid and reliable stability-indicating HPLC assay was developed for the determination of ASL concentrations to ensure the accuracy of the data and the chemical stability of the liposomes was also determined.

2. Experimental

Asulacrine isethionate salt (CI-921, 99% pure) was synthesized and gifted by Auckland Cancer Society Research Centre, University of Auckland. The phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine monohydrate (DPPC), was purchased from Lipoid GmbH (Ludwigshafen, Germany), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) from Avanti Polar Lipids, Inc. Cholesterol (99%) was obtained from Sigma-Aldrich (Auckland, New Zealand). Poloxamer 188 (MW 7600 to 9510) was kindly donated by BASF New Zealand Limited (Auckland). Acetonitrile (HPLC grade) and methanol were obtained from EMD Millipore Corporation, USA. Water purified on a Milli-Q system (Millipore, USA) was used. All other chemicals were of analytical grade from EMD Millipore Corporation, USA.

2.1. Stability-indicating HPLC method development and validation

2.1.1. Instrumentation and chromatographic conditions

The Agilent Technologies 1200 series HPLC system was equipped with a G1311A quaternary pump, G1329A auto-sampler (set at 4 °C) and a G13150 diode array detector. The mobile phase consisted of 20 mM monopotassium dihydrogen phosphate (KH_2PO_4) buffer (pH 2.5) and acetonitrile (72: 28, v/v). The mobile phase was filtered through 0.22 μm nylon filter and degassed in an ultrasonic bath before use. The autosampler temperature was maintained at 25 °C and 20 μl was injected into HPLC. All samples were pumped at a flow rate of 1.0 mL/min through a C18 Luna 5 μm, 150 × 4.6 mm column with UV detection at 254 nm. The output signal was monitored and processed using HPLC Chem Station Software.

2.1.2. Preparation of stock, standards and quality control (QC) samples

Stock solutions were prepared by dissolving ASL in methanol to give an 800 μg/mL solution. Analytical standards of ASL (0.06, 0.08, 0.1, 0.5, 1, 2 and 5 μg/mL, as isethionate salt) were prepared by further diluting the stock solutions with a mixture of acetate buffer (pH 3.8, 20 mM) and methanol (20/80, v/v). Quality control (QC) samples were prepared at 0.2, 1.5 and 4 μg/mL in the same way as described above from the working standard solutions. All solutions were stored at −20 °C until use.

2.1.3. Specificity and forced degradation of ASL

The stability-indicating nature of the assay was determined by analysis of the separation of the ASL peak from degradation peaks produced following exposure of ASL solutions to 10 mM HCl (pH 2), 10 mM NaOH (pH 12), 3% hydrogen peroxide (H_2O_2) for 24 h. The experiments were carried out as follows: a 800 μg/mL stock solution of ASL in methanol was mixed with HCl or NaOH or H_2O_2 solutions at 1: 19 (v/v) and placed in an oven set at 37 °C. Samples were taken at different time points depending on the decomposition rate, diluted with a quenching solution (to stabilize the sample) and analyzed immediately.

The specificity of the assay was determined by the complete separation of ASL in the presence of its degradation products generated under various stress conditions. The peak purity analysis for ASL was carried out on stressed samples using a diode array detector (Jain et al., 2009).

2.1.4. Linearity

The linearity of the assay was validated over the ASL concentration range of 0.06–5 μg/mL. Standard regression techniques were used to construct standard curves. All standard solutions were protected from light and analyzed immediately.

2.1.5. Precision and accuracy

Quality control (QC) standards, 0.2, 1.5 and 4 μg/mL were analyzed repeatedly ($n = 5$) during an analytical run then again over 3 consecutive days in order to determine intra-day and inter-day variability, respectively. Accuracy was evaluated by comparing the theoretical concentrations with the predicted concentrations of the QC standards using the calibration curve. Precision is reported as coefficient of variation (CV).

2.1.6. Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined based on the standard deviation (SD) of the response and the slope (S) of the calibration curves ($n = 3$) using 3.3(SD/S) and 10(SD/S), respectively.