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Profiling of dalcetrapib metabolites in human plasma by accelerator mass spectrometry and investigation of the free phenothiol by derivatisation with methylacrylate

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ABSTRACT

Dalcetrapib, a thioester prodrug, undergoes rapid and complete conversion in vivo to its phenothiol metabolite M1 which exerts the targeted pharmacological response in human. In clinical studies, M1 has been quantified together with its dimer and mixed disulfide species that represent the 'dalcetrapib active form' in plasma. In this article, we describe the determination of the free phenothiol M1 by derivatisation with methylacrylate as a percentage of 'dalcetrapib active form'. Pharmacokinetic profiles of M1 after oral administration of dalcetrapib to humans could be established, underscoring the validity to use a composite measure of 'dalcetrapib active form' as a surrogate marker for pharmacodynamic evaluations. 'Dalcetrapib active form' and M1 made up 8.9% and 3.6% of total drug-related material, respectively. In addition, complete metabolite profiling of ^{14}C-labeled dalcetrapib was conducted after two-dimensional HPLC using fast fractionation into 384-well plates and ultrasensitive determination of the ^{14}C-content by accelerator mass spectrometry. M1 underwent further biotransformation to its S-methyl metabolite M3, which was further oxidized to its sulfoxide and sulfone. Another metabolic pathway was the formation of the S-glucuronide. All of these species underwent further oxidation in the ethylbutyl cyclohexyl moiety leading to a multitude of hydroxyl and keto metabolites undergoing further conjugation to O-glucuronides. More than 80 metabolites were identified, demonstrating extensive metabolism. However, it was unambiguously demonstrated that none of these metabolites were major according to the MIST guideline (exceeding 10% of drug related material in circulation). The combination of accelerator mass spectrometry with HPLC together with high resolution mass spectrometry allowed for structural characterization of the most relevant human metabolites.

1. Introduction

Dalcetrapib is an inhibitor of the cholesteryl ester transfer plasma protein (CETP) under development for the prevention of cardiovascular disease [1,2]. The thioester of dalcetrapib undergoes rapid hydrolysis in vivo to its thiophenol metabolite (M1) which reversibly binds to cysteine 13 of CETP inhibiting its cholesteryl ester transfer function [3,4]. For this reason, dalcetrapib acts as a prodrug, with the free thiophenol metabolite (M1) as the pharmacologically active form. However, in vivo, the thiophenol also undergoes further oxidation to form its dimer (M2) or mixed disulfides (Mx) with e.g. glutathione or cysteine-rich proteins. Thus, it may be expected that M1 co-exists in a dynamic oxidation-reduction equilibrium between all of these species.

Since the development and application of a bioanalytical assay to determine the free portion of the thiophenol metabolite (M1) proved to be very challenging, these individual forms were determined collectively as 'dalcetrapib active form' by reduction of the disulfides with dithiothreitol (DTT) and derivatization with N-ethylmaleimide (NEM) to produce the stable NEM derivative of the thiophenol metabolite (M1) [5]. This analytical assay provided

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the robustness and throughput needed for application to support pharmacokinetic and pharmacological analysis of dalcetrapib in humans for regulatory purposes.

In a previous first human mass balance study of dalcetrapib, the sensitivity of the radio-HPLC method was limited, presumably due to the extensive metabolism. Therefore, a second mass balance study was conducted dedicated to the elucidation of the human metabolism of dalcetrapib. To exclude the presence of a major metabolite (>10% of total radioactivity) from a 'metabolite in safety testing' (MIST) perspective [7,8], methods for the analysis of human plasma samples were further optimized. A two-dimensional HPLC method was combined with fraction collection into 384-well plates and highly sensitive ^{14}C-content analysis using accelerator mass spectrometry (AMS) to establish high resolution radiochromatograms.

The AMS technology is based on sample graphitization followed by combustion and detection of the ratio between ^{14}C and ^{12}C by accelerating ions to extraordinary high kinetic energies before mass analysis. Developed in the late 1970s, AMS became applicable to ^{14}C analysis in pharma industry some decades later. Since AMS has a much higher sensitivity compared to conventional LSC, it is an attractive option for pharma industry offering unique possibilities for the analysis of low tracer amounts (^{14}C) in human PK and ADME studies [9].

In this article, we describe the investigation of circulating plasma metabolites of dalcetrapib. There were three main goals of this investigation: I. to determine the exposure of the free thiophenol of dalcetrapib in plasma which solely exerts the pharmacological response by its binding to cysteine 13 of the endogenous CETP protein. II. to determine whether a major circulating metabolite exists exceeding 10% or more of total drug-related material [6–8]. III. to elucidate the structures of the detected metabolites.

2. Materials and methods

2.1. Reference compounds

The test compound was dalcetrapib (RO4607381-003; Lot RC09104185), and the radioactive compound was ^{14}C-labeled dalcetrapib (batch number GSA0180/01) provided by F. Hoffmann-La Roche AG. Both compounds were mixed to a formulation containing 600 mg dalcetrapib resulting in a specific radioactive activity of 6.67 kBq/mg corresponding to 180 nCi/mg.

2.2. Chemicals, reagents and materials

Formic acid, 98–100% (Suprapur) and ethanol (Lichrosolv) were purchased from Merck (Darmstadt, Germany). Acetonitrile (HPLC grade S) was obtained from Rathburn (Walkerburn, U.K.). Water used for chromatography was Lichrosolv grade from Merck. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was obtained from Thermo Scientific (Zürich, Switzerland) as solution at neutral pH, Methylacrylate was supplied by Sigma-Aldrich (Buchs, Switzerland). General laboratory reagents and solvents were of analytical or HPLC grade and were supplied by Fluka or Sigma-Aldrich (Buchs, Switzerland) and Merck AG (Darmstadt, Germany). For AMS analysis, the following reagents were used: Methanol (Fisher Scientific, Loughborough, United Kingdom), Sodium benzoate (Sigma-Aldrich Chemical Co, Gillingham, United Kingdom), Graphitization tubes (York Glassware Services Ltd, York, United Kingdom), Sample tubes (York Glassware Services Ltd), Combustion tubes (York Glassware Services Ltd), Borosilicate glass tubes (York Glassware Services Ltd), Copper oxide wire (ACS) (Sigma-Aldrich Chemical Co), Cobalt powder (100 Mesh, 99.9%) (Sigma-Aldrich Chemical Co), Zinc powder (100 Mesh, 99%) (Sigma-Aldrich Chemical Co), Titanium (II) Hydride (325 Mesh, 98%) (Sigma-Aldrich Chemical Co), Aluminium cathode (National Electrostatics Corp, USA), ANU sugar (certificated value = 1.5061 Times Modern) (Quaternary Dating Research Centre, Australian National University, Canberra, Australia), Synthetic graphite 200–325 Mesh, (99.9999%) (Alfa Aesar from Johnson Matthey PLC Acros Organics), Solid aluminium cathode (Machine blank) (National Electrostatics Corp, USA).

2.3. Clinical study

This was a non-randomized, open-label, one-treatment, one-period design study conducted at a single centre in The Netherlands (PRA International, Zuidlaren) in 2011. Study protocols were approved by the appropriate responsible authorities and independent ethics committees, and all study participants provided written informed consent. The studies were conducted in accordance with good clinical practice guidelines and ethical standards for human experimentation established by the Declaration of Helsinki. The metabolism of dalcetrapib was investigated in human volunteers after oral administration of 600 mg of partially [^{14}C] labeled dalcetrapib (equivalent to ~4.0 MBq). A detailed description of the study including pharmacokinetic parameters will be described in a manuscript entitled "Absorption and disposition of dalcetrapib, a thioester prodrug of a thiol, in humans" by Darren Bentley et al. that is currently in preparation.

2.4. Preparation of proportionate plasma pools

Proportionate plasma pools (individual AUC pools) were prepared from each subject in the study covering the sampling times 0–24 h according to the procedure of Hamilton et al. [10,11], corresponding to four times T_{max}. The proportion of each metabolite in this sample, determined by radioactivity measurement in comparison to total radioactivity, directly returns the proportion of $AUC_{0\text{-}24h}$ of each metabolite to that of total radioactivity. Consequently, the pooling of the individual AUC pool samples of all 6 subjects provides one sample from which the average proportion of each metabolite in terms of total radioactivity $AUC_{0\text{-}24h}$ can be determined. In this report, this sample is referred to as the 'AUC plasma pool sample'.

2.5. Sample preparation for analysis

Dalcetrapib and its metabolites were isolated from plasma (1 mL) by adding 20 μL of methylacrylate and vortexing. Then 3.0 mL of ethanol were added and vortexed for ca. 10 s. Thereafter, 10 μL of 0.5 M TCEP were added and vortexed to mix, then sonicated for 10 min. The samples were further vortexed at room temperature for 30 min, and then centrifuged at about 16000*g* for 5 min to achieve protein precipitation of plasma. The isolated supernatant (ca. 4 mL) was evaporated to a volume of about 1.7 mL using a vacuum centrifuge kept at 50 °C. An aliquot (1 mL) of this solution was injected onto the trapping column of the column-switching HPLC system of method 1 (see below).

The effluent of the trapping column of method 1 was collected for 7 min during the loading procedure. The volume collected was 17.5 mL. The solvent of this sample was evaporated for 30 min using a vacuum centrifuge kept at 50 °C. The final volume was then about 13 mL, to which 11 mL of 1% aqueous formic acid was added. The whole sample (24 mL) was transferred to an OASIS HLB cartridge (Waters) for solid phase extraction of the radioactive drug-related material. Prior to use, the OASIS cartridges were washed with 2 mL of methanol followed by 2 mL of water before the sample was loaded. Thereafter, the cartridge was washed with 0.2% formic