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Determination of dalcetrapib by liquid chromatography–tandem mass spectrometry

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ABSTRACT

The cholesteryl ester transfer protein modulator dalcetrapib is currently under development for the prevention of dyslipidemia and cardiovascular disease. Dalcetrapib, a thioester, is rapidly hydrolyzed *in vivo* to the corresponding thiophenol which in turn is further oxidized to the dimer and mixed disulfides (where the thiophenol binds to peptides, proteins and other endogenous thiols). These forms co-exist in an oxidation–reduction equilibrium *via* the thiol and cannot be stabilized without influencing the equilibrium, hence specific determination of individual components, *i.e.*, in order to distinguish between the free thiol, the disulfide dimer and mixed disulfide adducts, was not pursued for routine analysis. The individual forms were quantified collectively as dalcetrapib-thiol (dal-thiol) after reduction under basic conditions with dithiothreitol to break disulfide bonds and derivatization with N-ethylmaleimide to stabilize the free thiol. The S-methyl and S-glucuronide metabolites were determined simultaneously with dal-thiol with no effect from the derivatization procedure. Column-switching liquid chromatography–tandem mass spectrometry provided a simple, fast and robust method for analysis of human and animal plasma and human urine samples. Addition of the surfactant Tween 80 to urine prevented adsorptive compound loss. The lower limits of quantitation (LLOQ) were 5 ng/mL for dal-thiol, and 5 ng/mL for the S-methyl and 50 ng/mL for the S-glucuronide metabolites. Using stable isotope-labeled internal standards, inter- and intra-assay precisions were each <15% (<20% at LLOQ) and accuracy was between 85 and 115%. Recovery was close to 100%, and no significant matrix effect was observed.

1. Introduction

Dalcetrapib (RO4607381; JTT-705), which targets cholesteryl ester transfer protein (CETP) activity and increases high-density lipoprotein cholesterol, is in Phase III clinical development to assess its impact on atherosclerosis and cardiovascular outcomes [1]. Dalcetrapib (dal-ester), a thioester, undergoes rapid hydrolysis by nonspecific esterases in biological fluids and in the intestine to generate the pharmacologically active free thiol which binds to CETP (data on file). It is well known that sulfhydryl-containing peptides, proteins and low molecular weight compounds (such as glutathione or cysteine) exist in the body as mixed disulfides [2]. Oxidation of thiol drugs to form disulphide bonds either with itself or endogenous thiols has been reported for tiopronin [3], clopidogrel [4] or captopril [5]. Similarly, dalcetrapib free thiol is expected to form dimers and mixed disulfides with endogenous thiols, and binds to plasma proteins ('active form' dalcetrapib-thiol [dal-thiol]) (Fig. 1). Disulfide dimer and endogenously bound thiol are considered to co-exist in body fluids in oxidation–reduction equilibrium *via* the free thiol. In animals, dal-thiol undergoes extensive metabolism *via* glucuronic acid conjugation, methylation and hydroxylation (but not sulfation) to form pharmacologically inactive S-methyl (dal-S-Me) [6] and S-glucuronide (dal-S-Glu) metabolites [7], which are further metabolized to a variety of secondary oxidation products. In a mass balance study of orally administered dalcetrapib in healthy male volunteers [8], dal-thiol was the principal component in human plasma, while pharmacologically inactive dal-S-Me and dal-S-Glu were the most abundant metabolites.

An analytical method was needed to allow determination of drug exposure in non-clinical pharmacokinetic and toxicokinetic studies as well as in clinical studies. Specifically, a reliable measurement of free, bound, or the sum of all forms and metabolites had to be established. Liquid chromatography–tandem mass

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spectrometry (LC–MS/MS) was selected as the available method of choice to provide selective and sensitive bioanalysis. Thiol compounds typically present challenges for bioanalysis due to their high reactivity and need to be stabilized before measurement, for example, by pH adjustment or derivatization with alkylating agents such as maleimides or methylacrylate. Disulfides are usually converted to thiols by treatment with reducing agents (*e.g.*, dithiothreitol (DTT) or tributylphosphine) and derivatized for analysis. Comprehensive summaries of analytical procedures for thiols and disulfide can be found in previous literature reviews [9–11]. Simultaneous analysis of thiols and disulfides using differential labeling has been described [11,12], but these laborious multi-step procedures present practical difficulties for the routine analysis of large numbers of samples during drug development. This manuscript describes the development and validation of a column-switching method using LC–MS/MS to determine thioester, free thiol, disulfide dimer, and mixed disulfides as combined 'active form' (dal-thiol, as N-ethylmaleimide [NEM] derivative), and principal metabolites. The method was further refined to facilitate routine handling of large sample batches from clinical and preclinical studies during drug development, and to permit simultaneous analysis of total 'active form' (dal-thiol) and dal-S-Me and dal-S-Glu metabolites. Stability of dalcetrapib thioester in biological media was investigated and the equilibrium status of various components of dalcetrapib 'active form' and the potential for their quantitation are discussed; in this regard, the sum of 'active form' (dal-thiol) following thiolysis with DTT and alkylation with NEM was selected for routine quantification, with simultaneous measurement of the dal-S-Me and dal-S-Glu metabolites. Findings are presented following method validation based on internationally accepted guidelines and application of the method to animal and human study samples.

2. Experimental

2.1. Chemicals, solutions and standards

Ethanol (Lichrosolv for HPLC) was obtained from Merck (Darmstadt, Germany), and acetonitrile (HPLC grade S) from Rathburn (Walkerburn, U.K.). Ammonium formate (p.a.), formic acid 98–100% (Suprapur grade) and Tween 80 were purchased from Fluka (Buchs, Switzerland). DTT was purchased from AppliChem (Darmstadt, Germany) and NEM from Acros Organics (Geel, Belgium). Solutions of 0.1N NaOH and HCl were prepared from Merck Titrisol ampoules. Water used for preparation of all solutions was from a Milli-Q apparatus (Millipore, Billerica, MA, USA).

Dalcetrapib (dal, MW 389.6) and isotopically (D_{10} at cyclohexane ring) labeled dalcetrapib used as internal standard (dal-IS, MW 399.66) and the NEM derivative reference dal-NEM (MW 444.64) were provided by F. Hoffmann-La Roche Ltd. (Basel, Switzerland). The free thiophenol of dalcetrapib (thiol, MW 319.51), thiophenol dimer (dimer, MW 637.01), dal-S-Me (MW 333.54) and dal-S-Glu (MW 496.24) were obtained from Japan Tobacco Inc. (Osaka, Japan). Stable isotope labeled dal-S-Me ($^{13}CD_3$ at methyl group, MW 337.55) and dal-S-Glu (D_{10} at cyclohexane ring, MW 505.70) used as internal standards were synthesized at F. Hoffmann-La Roche. Stock solutions of analytes (1 mg/mL) were prepared in ethanol and further diluted with ethanol to give spiking solutions. Small aliquots of spiking solutions were added to blank plasma or to blank urine containing 0.2% Tween 80 for the preparation of calibration standards and quality control (QC) samples: Method 1, 5–4000 ng/mL dal-thiol; 5–4000 ng/mL (5–2000 ng/mL, Method 2) dal-S-Me; 50–10,000 ng/mL (50–4000 ng/mL, Method 2) dal-S-Glu. Calibration standards and QC materials were prepared freshly before use. Internal standard stock solution (1 mg/mL dal-IS) was prepared in ethanol and further diluted to a 200 ng/mL working solution.

A stock solution of DTT in water was prepared at 23.14 mg/mL (150 mM). DTT working solution was obtained by mixing 5 mL DTT stock solution, 20 mL 0.1 N NaOH and 75 mL ethanol/water 70:30 (v/v). A NEM stock solution of 125 mg/mL (1 M) was prepared in acetonitrile; the NEM working solution comprised 5 mL NEM stock solution, 20 mL 0.1 N HCL and 75 mL ethanol. Reagent stock solutions were stable for at least 1 month and working solutions for up to 1 week if stored chilled.

Blank animal plasma was prepared either from EDTA or heparin blood (rat, mouse, hamster, cynomolgus monkey and rabbit) in laboratories at F. Hoffmann-La Roche. Human heparin plasma was purchased from TRINA Bioreactives (Greifensee, Switzerland). Urine was supplied by donors from our own laboratories. Human gastrointestinal fluids were collected in the Roche Clinical Pharmacology Unit (Strasbourg) under fasted or stimulated conditions.

2.2. Sample preparation

2.2.1. Derivatization procedure with DTT/NEM for dal-thiol

To 50 μL plasma or urine, 300 μL ethanol (double blank only) or internal standard solution was added for protein precipitation, followed by 100 μL of DTT working solution. After vortex-mixing (Heidolph model Reax 2000; Heidolph Instruments, Schwabach, Germany), the sample solution was left to react for approximately 30 min at room temperature. For derivatization, 100 μL of NEM working solution was added. After vortex-mixing and centrifugation (6 min at 15,000 rpm; 10 °C, Heraeus Multifuge 3 S-R, Thermo Electron LED, Zürich, Switzerland), supernatant was transferred to the injection vial.

2.2.2. Protein precipitation (without derivatization)

To investigate the stability of dalcetrapib thioester, 10 volumes ethanol was added to plasma after the desired storage time, mixed, centrifuged (6 min at 15,000 rpm) and supernatant was transferred into a new vial. For free thiol or dimer analysis, ethanol or acetonitrile was used for protein precipitation; the ratio of plasma to solvent ranged from 1:2 to 1:10 (v/v).

2.2.3. Derivatization with NEM before protein precipitation

5 μL aliquots of 1 M NEM stock solution were added to 50 μL plasma, followed by protein precipitation (*i.e.* with 500 μL of ethanol).

2.2.4. Derivatization with NEM after protein precipitation

Organic solvent (*i.e.* 500 μL of ethanol) was added to 50 μL plasma, followed by addition of 5 μL of 1 M NEM stock solution.

2.2.5. Pretreatment with DTT (without NEM derivatization)

Procedures as described in Section 2.2.1 but with addition of 100 μL of ethanol instead of NEM solution.

2.3. Liquid chromatography–mass spectrometry

2.3.1. Method 1

A schematic of the column-switching LC–MS/MS system is shown in Fig. 2 and details of the LC program are provided in Table 1.

The following conditions were used to optimize sample preparation, to investigate the feasibility of determining thioester and entities of the 'active form' (free thiol, dimer, adducts) separately and to perform initial stability tests. Furthermore, the method served for quantitation of dal-thiol in plasma from clinical and preclinical studies and for exploratory determination of metabolites dal-S-Me and dal-S-Glu. Sample solution (5–50 μL)