Bioanalytical Method Development for Drugs Associated with Issues Derived from Conversion of Compounds

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ABSTRACT

Quantification of drugs and their metabolites in animal biological fluids such as plasma and urine is a common practice in drug development within the pharmaceutical industry. As drug concentrations in biological fluids play a critical role in assessment of drug safety and efficacy, bioanalytical methods are required to be accurate and reproducible.

Bioanalytical issues of chemosynthetic drugs, a frequent target of research and development in Japan, have been studied by many scientists. Among the issues, questions remain about the conversion of drugs and their metabolites. This is despite the considerable adverse impact of conversion issues on the accuracy and reproducibility of concentration data. The conversion issues are divided into Total Concentration Issue and Individual Concentration Issue. Total Concentration Issue is an issue a laboratory researcher encounters if the total concentrations of an analyte before and after conversion are determined, and Individual Concentration Issue is a group of issues a laboratory researcher faces if the concentration to be measured is an individual concentration (not total concentration) and the effect of conversion on analyte quantification has to be excluded. Individual Concentration Issue is further divided into two issues. Analyte Decrease Issue is an issue a laboratory researcher faces if the concentration of an analyte is observed to decrease and Non-analyte Interference Issue is an issue when the researcher manages the risk of overestimation of an analyte concentration due to the conversion of non-analytes (for example, metabolites of the analytes) to the analyte.

Total Concentration Issue is recognized if a drug has conformers and the conformers are analyzed as a single component. Conformers form a bimodal peak under most HPLC conditions. This peak shape hinders establishment of the necessary lower limit of quantification and sufficient reproducibility. In addition, conversion during pretreatment and differences in physicochemical properties between conformers present additional difficulties

in the development of a reproducible pretreatment method.

Analyte Decrease Issue is recognized when an attempt is made to stabilize an analyte to preclude the underestimation of analyte concentration due to conversion (including degradation) of analytes. While many stabilization methods have been reported, only a few will be effective for any individual analyte. This situation impedes the development of stabilization methods.

Non-analyte Interference Issue is recognized when presence and stability of non-analytes in a study sample are examined in order to mitigate the risk of overestimation of analyte concentration due to conversion of non-analytes. Most laboratories do not extensively examine this potential interference from non-analytes, as it requires additional time and energy in method development, and is not required by regulatory agencies.

In this research, to solve these three issues, the author constructed and applied three "workflows" (one workflow per issue) to three new drug candidates, ASP2151, ASP3258, and enzalutamide. "Workflows" are figures in which tasks such as experiments and data interpretation are placed in a proper order, and are prepared to solve issues effectively. The author constructed a workflow for the determination of the total concentration of conformers, which streamlines development of a pretreatment method and an HPLC method under which conformers form a single peak. The author then constructed a workflow for the stabilization of a convertible analyte in which the cause of conversion is estimated from analyte chemical structures etc. and used to prioritize stabilization methods to be examined experimentally. Finally, the author constructed a workflow for interference from convertible non-analytes to assess the possibility of interference in the early phase of method development by using available information including chemical structures of dosed drugs and analytes, and to accordingly take measures depending on the possibility of interference.

When the workflow for the determination of the total concentration of conformers

was applied to ASP2151, an HPLC method and a pretreatment method were developed smoothly. The method validation results demonstrated that the method has the necessary lower limit of quantification and sufficient reproducibility. When the workflow for the stabilization of a convertible analyte was used in method development for enzalutamide metabolite M2, the workflow helped to determine that hydrolysis of an amide bond of M2 could be stopped by the cooling of samples and addition of an esterase inhibitor. The workflow for interference from a convertible non-analyte was applicable for ASP2151, ASP3258, and enzalutamide and facilitated the employment of measure consistent with the possibility of interference. No interference from a non-analyte was observed in sample analysis, demonstrating that the workflow can identify and solve issues during method development using fewer resources than the currently available recommendation. Finally, the developed bioanalytical methods were applied to sample analysis of ASP2151, ASP3258, and enzalutamide and clarified absorption and concentration-time profiles.

The three workflows established in the present research are expected to aid drug development, by solving all issues derived from the conversion of compounds and facilitating the development of accurate and reproducible bioanalytical methods. In addition, absorption and concentration-time profiles of ASP2151, ASP3258, and enzalutamide in animals were determined to support the development of these drug candidates.

CONTENTS

ABBREVIATIONS

α	Separation factor
AG	Acyl glucuronide
APCI	Atmospheric pressure chemical ionization
AUC	Area under the plasma concentration-time curve
BA	Bioavailability
C_{max}	Maximum concentration
CRPC	Castration-resistant prostate cancer
CSV	Comma-separated value
CV	Coefficient of variation
DDVP	2,2-Dichlorovinyl dimethyl phosphate
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase IV
ED_{50}	50% effective dose
ESI	Electrospray ionization
FDA	Food and Drug Administration
HPLC	High-performance liquid chromatograph or High-performance liquid chromatography
HPLC-FL	High-performance liquid chromatograph-fluorescence detector or High-performance liquid chromatography-fluorescence detection
i.v.	Intravenous
ICH	International Council for Harmonisation
IS	Internal standard

ABBREVIATIONS (continued)

k	Retention factor
LC-MS/MS	Liquid chromatograph-tandem mass spectrometer or Liquid chromatography-tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
M1	Carboxylic acid metabolite of enzalutamide
M2	*N*-desmethyl enzalutamide
MOA	Mechanism of action
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
N	Theoretical plate number
QC	Quality control
R^2	Coefficient of determination
RE	Relative error
S	Symmetry factor
SPE	Solid phase extraction
$t_{1/2}$	Elimination half-life
TBME	*tert*-Butyl methyl ether
THF	Tetrahydrofuran
T_{max}	Time to reach maximum concentration
Vd_{ss}	Volume of distribution at steady state

CHAPTER 1 GENERAL INTRODUCTION

Quantification of drugs and their metabolites in animal biological fluids such as plasma and urine is a common practice in drug development within the pharmaceutical industry, with the aim of clarifying the absorption, distribution, metabolism, and excretion of a drug in drug development, and determining the drug concentration at which efficacy and side effects are detected. In relation to this, the United States Food and Drug Administration (FDA) issued guidance about the performance verification (validation) of bioanalytical methods in 2001 (FDA, 2001). In accordance with the FDA guidance, it is not allowed to apply a method to study samples immediately after the method is developed. Before application to study samples, a validation study has to be conducted in order to confirm that the method meets the required acceptance criteria in terms of selectivity, accuracy (closeness of a measurement to the true value), precision (reproducibility), and stability, etc. In addition, it is necessary to solve issues, if any, that affects the accuracy and reproducibility of analytical results, even if the issue is not listed in the guidance. If a pharmaceutical company measures drug concentrations using unreliable methods and submits the concentration data to regulatory agency to obtain approval for a pharmaceutical product, the product will not be approved by the regulatory agency and therefore will not be used in medical care. Given that, it is critical to develop reliable bioanalytical methods in drug development.

Chemosynthetic drugs are one of the major drug classes and a frequent target of research and development in Japan. Generally, bioanalytical methods for chemosynthetic drugs start with pretreatment, such as protein precipitation, liquid-liquid extraction (LLE), solid phase extraction (SPE), or column-switching etc. The resulting samples (extracts) are injected onto a high-performance liquid chromatograph (HPLC) to separate an analyte of interest from other components and then to detect the analyte. Among detection methodologies, ultraviolet and fluorescence have conventionally been used, but tandem mass

spectrometry (MS/MS) has come to be more frequently employed. This equipment and the trained technicians required to use it are commonly available at research facilities dedicated to drug development, and it is now feasible for research facilities to analyze many samples with high reliability.

Bioanalytical method development for chemosynthetic drugs has been studied by many scientists (Bakhtiar & Majumdar 2007; Hendriks 2009; James et al. 2004; Meng et al. 2013; Jemal et al. 2010; Unger et al. 2015). A bioanalytical method is typically established as follows. When a laboratory researcher plans to analyze samples in accordance with the FDA guidance, it is very likely that the drug candidate has already completed the early drug discovery stage. Given this, the researcher can initiate development of the method by collecting information such as light sensitivity, solubility, existence of isomers, *in vitro* metabolism data, mechanism of action (MOA), a dosage form, and potential internal standard (IS) material. Then, the researcher uses reference standard material of the analyte to establish a detection method with adequate linearity and the necessary lower limit of quantification (LLOQ). Then, the researcher experimentally finds an HPLC method which provides moderate retention, a good peak shape, short run time, and negligible carryover. At this stage, the researcher may examine adsorption of the analyte to containers. Then, the researcher spikes the analyte in blank biological fluids and investigates a pretreatment method. The pretreatment method is expected to show consistent recovery over a broad range of analyte concentrations, and provide adequately clean samples that can be analyzed continuously for weeks without soiling the analytical instrument. Then, before conducting a validation study, the researcher may confirm whether or not the method can pass validation requirements according to the FDA guidance. As a “formal” validation study involves extensive documentation including raw data, a protocol, and a report, if the result in the “formal” validation study does not meet the validation requirements, much time and energy will be lost.

To circumvent this, it is a common practice to perform a preliminary experiment under the same study design as the “formal” validation study and to judge whether it is reasonable to move the method forward to a “formal” validation study. This preliminary experiment is called pre-validation. If the pre-validation result does not meet the validation requirements, the researcher will modify the method to solve the issue, and conduct the pre-validation again. This cycle can be repeated more than once. When the researcher obtains an acceptable pre-validation result, a “formal” validation study will be started and followed by study sample analysis. Unfortunately, the processes mentioned above do not always go smoothly. Major issues are listed in Table 1-1. To circumvent these issues, the author considers it necessary to add or change an experimental item in method development and streamline the experimental items.

Among the issues listed in Table 1-1, those associated with conversion of analytes, dosed drugs, and their metabolites appear to need more investigation; the author has often observed unpleasant incidents in which the issue was overlooked or required too long time to solve. In addition, conversion issues are regarded as important in the literature because they deteriorate the accuracy and reproducibility of concentration data and therefore hinder drug development. Given this, the author considers it definitely meaningful to study conversion issues and provide solutions to them. The conversion issues are divided into Total Concentration Issue and Individual Concentration Issue (Fig. 1-1). Total Concentration Issue is an issue a laboratory researcher encounters if the total concentrations of an analyte before and after conversion are determined, and Individual Concentration Issue is a group of issues a laboratory researcher faces if the concentration to be measured is an individual concentration (not total concentration) and the effect of conversion on analyte quantification has to be excluded. Individual Concentration Issue is further divided into two issues. Analyte Decrease Issue is an issue a laboratory researcher has if concentration of an analyte is observed to

decrease and Non-analyte Interference Issue is an issue a laboratory researcher has when the researcher manages the risk of the overestimation of an analyte concentration due to the conversion of non-analytes (for example, metabolites of the analytes and dosed drugs) to the analyte. In the rest of "General Introduction", background of the three issues and necessity of solutions to the issues will be explained. Then, three drug candidates that will be used to assess the proposed solutions in the present research will be introduced. At the end of "General Introduction", the aim of the present study will be clarified.

1.1 Issue associated with determination of total concentration of a convertible analyte

To the author's knowledge, examples of determining the total concentration before and after conversion are limited to conformer interconversions. Conformers are a type of isomer, and are observed because the compound cannot alter its conformation (or rotate its moiety) freely. In other words, conformers do not exist when conformation alternation can occur freely. A nomenclature for conformers has not been established, but one can call them the *E* form and the *Z* form, following the *E/Z* convention adopted by International Union of Pure and Applied Chemistry. According to LaPlante (Laplante et al., 2011), if a drug candidate has conformers, the pharmaceutical company will take one of the two options depending the interconversion rates. If conformer interconversion is hindered severely and takes more than one year, it is reasonable to measure the concentrations of the *E* form and *Z* form separately. In contrast, if the conformer interconverts to its isomer within the period from the time of administration to the time of excretion out of the body, it is necessary to take another option. As the interconversion is fast, it is technically difficult to distinguish the *E* form and *Z* form in *in vivo* efficacy and safety evaluation. In addition, it is not feasible to determine the individual concentration of the *E* form and *Z* form *in vivo* because the conformers interconvert considerably during sample collection, storage, and analysis.

Therefore, the option to take is to regard the *E* form and *Z* form as a single component in drug development and to measure the total concentration in biological fluids without separating the two conformers.

It is known that most of conformers form an ugly bimodal peak on an HPLC chromatogram (see Fig. 1-2 as an example of enalaprilat) and it is speculated that this peak shape is formed because of interconversion during HPLC analysis. Although several detection methods not involving HPLC separation have been reported (Chernetsova and Morlock, 2011; Jackson et al., 2010; Kovarik et al., 2007; Wagner et al., 2008; Yu et al., 2009), these methods are not as suitable for use in regulated bioanalysis as liquid chromatography-tandem mass spectrometry (LC-MS/MS), due to issues such as assay variability, ion suppression/enhancement, and selectivity. In addition, given the author's goal of establishing a bioanalytical method to support the development of drug candidates, the method should be based on a methodology familiar to drug development test facilities (i.e. LC-MS/MS).

When using LC-MS/MS under conditions which allow the two conformers to form unseparated broad peaks, there are two options with respect to analyte response for quantification. The first option is to use only the *E* form peak and ignore the rest of the analyte response in quantification. Assume that a ratio of *E* and *Z* form changes from 100:0 to 90:10 according to temperature and solvent. With this option, conformer ratios in injection samples directly affect quantification, resulting in an assay variation of approximately 10% due to variation in the conformer ratios. Given that regulatory acceptance criteria for precision and accuracy are 15%, other sources of variation must be markedly lower than usual, which is considered unfeasible. In addition, even if methods meet regulatory acceptance criteria, regulatory agencies may still not accept systemic errors of 10%. In addition, if the ratio of the two conformers varies more greatly (this is the case in prodigiosin derivative PNU-156804 (Fig. 1-3); the ratio varies from approximately 20:80 to 80:20 (Rizzo et al., 1999)), this option

is more problematic. For these reasons, the first option is not considered a general solution. The second option is to use the sum of the peak areas of the *E* and *Z* forms. Unfortunately, the peak of the minor conformer is often too small for detection at the LLOQ. In addition, if one injects a large amount of analytes to have a quantifiable peak area for the minor conformer at the LLOQ, it is very likely to have a very short range of calibration curve, as injection of a large amount of analytes will lead to saturation at a high concentration range. For these reasons, the second option is also not considered a general solution. Given these limited options, the utilization of LC-MS/MS and exploration of HPLC conditions under which conformers form a single peak is considered to be a reasonable approach to establishing bioanalytical methods for conformers in drug development. Scientists have been making strong efforts to have a single peak for their analyte of interest. A single peak was formed for cyclosporine (Bowers and Mathews, 1985), bisthiazolium derivatives (Nicolas et al., 2005), enalaprilat (Bouabdallah et al., 2003) by the use of a high column temperature, while a single peak was observed for prodigiosin derivative PNU-156804 by changing the mobile phase pH (Rizzo et al., 1999). However, to the author's knowledge, no general workflow has been proposed to have a single peak of conformers. As a result, bioanalytical method development remains a hurdle in the development of conformer-associated drug candidates. Based on their consideration of several hurdles, Clayden et al. recommend that development be restricted to conformer-free drug candidates (Clayden et al., 2009). However, if a bioanalytical method for conformers could be developed easily, it would allow pharmaceutical companies to develop new drug candidates without the constraint of the conformer issue in terms of bioanalysis. This is meaningful in drug design because some drugs with conformers have provided patients with valuable medical solutions.

In addition, pretreatment method development for conformers has not been studied extensively, despite a number of issues requiring consideration. Conformers have different

physicochemical properties to corresponding isomers, as indicated by differences in HPLC retention times under most HPLC conditions. As it is generally known that even slight differences in physicochemical properties potentially lead to discernable extraction recovery alternation, it is likely that the *E* form exhibits different extraction recovery from the *Z* form. Assume that pretreatment for prodigiosin derivative PNU-156804 was established in which one conformer recovery was 40% and the second was 60%. In this case, total recovery of samples with a conformer ratio of 20:80 would be $40 \times 0.2 + 60 \times 0.8 = 56\%$, whereas total recovery of samples with a conformer ratio of 80:20 would be $40 \times 0.8 + 60 \times 0.2 = 44\%$. This recovery difference (56% versus 44%) could not be corrected even when a single peak is formed on HPLC analysis, which would in turn directly introduce unacceptable bias into concentration data. Given this, it is reasonable to pursue a general workflow for pretreatment method development in which conformer ratios are controlled during the pretreatment.

Without having general workflows for HPLC and pretreatment method development, a laboratory researcher who does not have experience with conformers may spend several months, as there are quite a few parameters that the researcher can try but which are not relevant to conformer issues. A general workflow will serve to save time and effort.

1.2 Issue associated with concentration decrease of an analyte

Contrary to cases in which the total concentration of conformers is measured, if the concentration to be measured is an individual concentration, it is necessary to pay attention to conversion. If an analyte converts to another compound during sample collection, storage, and analysis, the conversion will cause underestimation of the analyte concentration in the body. As the stability test is included in the FDA guidance (FDA, 2001), this issue is recognized widely, and discovery of the problem is easy.

However, there is room for improvement in the solution to this issue. Stabilization

methods depend heavily on the analyte, and many stabilization methods are reported in the literature (Briscoe and Hage, 2009; Dell, 2004); these include a shorter storage period, reduced temperature, addition of enzyme inhibitors, addition of organic solvents, addition of antioxidants, light protection, pH adjustment, and derivatization. Given this, development of a stabilization method often takes a long time. In one extreme example, it took longer than one year to stabilize a certain analyte (personal communication within the workplace of the author). From the author's point of view, finding an appropriate stabilization method within a reasonable time frame is a major issue in actual research activity in the pharmaceutical industry. In this context, Chen et al. prepared a workflow in 2005 (Chen et al., 2005), to which Briscoe et al. added a shorter storage time and light protection in 2009 (Briscoe and Hage, 2009) (Fig. 1-4). However, Briscoe's workflow requires the step-by-step testing of stabilization measures, meaning that the workflow requires conducting many experiments before the appropriate stabilization method is established. Based on these circumstances, the author speculated that the workflow of stabilization had a substantial room for improvement.

Causes of conversion are helpful information in finding an appropriate stabilization method, and can be estimated in many cases using chemical structures, physicochemical data, and MOA (Briscoe and Hage, 2009; Chen and Hsieh, 2005; Hilhorst et al., 2015; Li et al., 2011; van de Merbel, 2013). Esters and amides are prone to hydrolysis enzymatically and chemically in biological fluids (Fig. 1-5(a)). Cytidine derivatives can be degraded by cytidine deaminases (Fig. 1-5(b)). Thiols are likely to form disulfide bonds within the molecules or with endogenous components chemically (Fig. 1-5(c)). *N*-glucuronide (Fig. 1-5(d)) and *N*-oxide (Fig. 1-5(e)) may convert chemically through deconjugation and reduction, respectively. Phenols and alcohols can be oxidized chemically (Fig. 1-5(f)). An analyte having a stereoisomer may convert to its corresponding enantiomer (Fig. 1-5(g)) or diastereomer (including *E/Z* forms; (Fig. 1-5(h)) enzymatically or chemically. Lactones and their

hydroxycarboxylic acid forms are likely to interconvert enzymatically or chemically (Fig. 1-5(i)). Physicochemical data of the analyte may indicate the possibility of light sensitivity. If the analyte is an anticancer agent with MOA in which the high reactivity of the drug plays an important role in its efficacy, the analyte may form covalent binding with endogenous components. If the MOA of the analyte is dipeptidyl peptidase IV (DPP-IV) inhibition, the analyte will be prone to degradation by DPP-IV. As shown in Table 1-2, effective stabilization methods are known for each compound class.

The author considers it is possible to construct an efficient workflow in which a chemical structure, physicochemical data, and MOA are examined and effective stabilization methods for each compound class are tested. It is the author's opinion that the new workflow will save time and energy, and thereby make a significant contribution to drug development.

1.3 Issue associated with interference due to conversion of a non-analyte

If a non-analyte compound in *in vivo* samples converts to an analyte after sample collection and the concentration of the analyte (not the total concentration of the non-analyte compound and the analyte) is measured, it is necessary to pay attention to the conversion (Bakhtiar and Majumdar, 2007; Dell, 2004; Hilhorst et al., 2015; James et al., 2004; Jemal et al., 2010; Li et al., 2011). Otherwise, the analyte concentration can be overestimated.

One non-analyte compound that can convert to an analyte (e.g. interfering compounds) is a metabolite of the analyte. Whereas it is not an issue that the analyte is metabolized during the systemic circulation of the analyte in the body, it is an issue that the metabolite converts to the analyte after sample collection. It is possible to predict whether the metabolite of interest can be produced in the body. The prediction can be helped by an understanding of chemical structures and *in vitro* metabolism study results (Billings et al., 1977; Ikeda, 2010). On the other hand, the *in vivo* concentration of the metabolite is

determined not only by the generation of the metabolite, but also by the excretion and further metabolism of the metabolite. Given this, the metabolite predicted using *in vitro* metabolism study results is not always detectable *in vivo* (Ikeda, 2015).

Acyl glucuronides (AGs; Fig. 1-6(a)) are well-known metabolites with regard to this issue and excellent reviews are available (Faed, 1984; Shipkova et al., 2003; Spahn-Langguth and Benet, 1992). When a drug with a carboxylic acid moiety is administered to animals, AG may be produced in the body. AG can convert to its aglycone by hydrolysis or produce a migrated isomer of AG by moving a drug moiety to another hydroxyl group of the AG. The latter reaction explains the phenomena that an AG is often detected as multiple peaks on chromatograms. The conversion to produce an aglycone by hydrolysis must be controlled as this conversion directly leads to overestimation of the analyte concentration. Unfortunately, AG conversion is very likely to occur in intact biological samples at room temperature, although the stability of AG varies depending on aglycone moieties. From the author's experience, carboxylic acid drugs often generate AGs *in vitro*, but it is rare that AG concentration is high in *in vivo* plasma samples. This discrepancy between *in vitro* and *in vivo* observation can be partially explained by the tendency of AGs to be efficiently excreted into bile, on the basis of their profiles as good substrates of drug transporters (Zamek-Gliszczynski et al., 2006).

N-oxides (Fig. 1-6(b)) can be generated enzymatically by oxidation of a tertiary amine moiety (Horie and Yokoi, 2003), and reduced to an unchanged form chemically (Li et al., 2011). From the author's experience, there are many drugs with a tertiary amine moiety, but there are not many cases in which *N*-oxides are produced. On the other hand, published research articles (Dell, 2004; James et al., 2004; Li et al., 2011) agree that *N*-oxides are generally unstable. Therefore, it is considered necessary to pay due attention to *N*-oxides.

Interconvertible stereoisomers (Fig. 1-6(c) and Fig. 1-6(d)) are another class of

interfering compounds (Briscoe and Hage, 2009; Hilhorst et al., 2015; Jemal et al., 2002; Li et al., 2011; van de Merbel, 2013). Of interconvertible stereoisomers, conformers that interconvert within a few days are analyzed for total concentration, but other interconvertible stereoisomers are analyzed to determine the isomers separately. Conversion occurs chemically or enzymatically. From the author's experience, many drugs have stereoisomers within their chemical structures, but stereoisomers are seldom observed *in vivo*. Testa et al. summarized the effect of the substituent on the interconversion rate (Testa et al., 1993).

Ether *O*-glucuronides (Fig. 1-6(e)) are formed enzymatically from drugs with an alcohol or phenol moiety (Yoshimura, 2010). Sulfates (Fig. 1-6(f)) are formed enzymatically from drugs with an alcohol, phenol, and amine (including hydroxylamine) moiety (Ozawa, 2010). While most ether *O*-glucuronides and sulfates are stable, some of them can readily convert to their aglycones (Bakhtiar and Majumdar, 2007; Li et al., 2011; Meng et al., 2013). From the author's experience, drugs with an alcohol, phenol, or amine moiety are not always associated with the *in vivo* or *in vitro* presence of ether *O*-glucuronide or sulfate.

Lactones and their hydroxycarboxylic acid forms (Fig. 1-6(g)) interconvert chemically under physiological conditions (Merbel 2013). Examples include camptothecins (irinotecan and topotecan) and statins (simvastatin, pravastatin, and atorvastatin etc.). Lactone concentration is overestimated if the conversion from a hydroxycarboxylic acid form is not stopped. On the contrary, it is necessary to stop conversion from the lactone when the analyte of interest is a hydroxycarboxylic acid form. Chemical structures clearly indicate whether this interconversion occurs and whether the interfering compound is present *in vivo*.

If a precursor metabolite or dosed drug of an analyte is readily changed into the analyte after sample collection, this interferes with quantification of the analyte. Representative examples are amides and esters (Hilhorst et al., 2015; Jemal and Xia, 2006)(Fig. 1-6(h)). If the dosed drug is a prodrug that is an esterified form of a

pharmacologically active compound, the drug is designed to be hydrolyzed readily in the body to exert efficacy; therefore, the hydrolysis of the prodrug is very likely. When a metabolite is quantified using a validated method, it is likely that an extensive drug metabolism work would be completed and it is practically feasible to evaluate precursor metabolites of the analyte as potential interfering compounds.

N-glucuronides can be interfering compounds but most are very stable (Bakhtiar and Majumdar, 2007; Dell, 2004; Li et al., 2011). *N*-glucuronides are much less commonly observed in animals than in humans (Kaivosaari et al., 2011).

If the issue of interference from convertible compounds is overlooked in method development and discovered in sample analysis, the method development and formal method validation study have to be conducted again, in accordance with FDA guidance. It will take several months to finish these and provide correct concentration data for the study samples. This is a considerable loss in drug development. Therefore, several research articles recommend the examination of interference during method development (Bakhtiar and Majumdar, 2007; Dell, 2004; Hilhorst et al., 2015; James et al., 2004; Jemal et al., 2010; Li et al., 2011). However, the recommendation in the articles is to administer the drug to animals, collect *in vivo* samples, and use the samples during method development in order to discover the interference issue early. As the recommended process involves an animal experiment, it will add approximately ten days to an experiment period in method development for all analytes, and the cooperation of the animal experiment department will be needed. In addition, the FDA guidance does not mention the interference issue. Further, unnecessary animal experimentation should be avoided from the viewpoint of animal welfare. For these reasons, the recommended process does not spread widely, and the interference issue is often overlooked. It is the author's opinion that it might be possible to propose a more practical approach than those used in previous research. The basic concept is as follows. By making

use of the above-mentioned knowledge, it seems possible to estimate the risk of interference at the beginning of method development. If the risk is high, it seems reasonable to obtain and use *in vivo* samples, but if the risk is low, it seems better to avoid animal experiments. The author considers that it is feasible and meaningful to develop a new workflow, and to examine its validity via the application of the workflow to real method development.

1.4 ASP2151, ASP3258, and enzalutamide

Three drug candidates ASP2151, ASP3258, and enzalutamide will be used to assess the proposed solutions in the present research.

ASP2151 (amenamevir; Fig. 1-7(a)(b)) is a potent helicase-primase inhibitor of disease caused by infection with herpes simplex virus (Chono et al., 2010). The 50% effective dose (ED_{50}) of ASP2151 was determined to be twice daily administration of 1.9 mg/kg in a mouse model (Chono et al., 2010). The compound exhibited promising efficacy in a Phase II trial (Tyring et al., 2012). In further drug development of ASP2151, the conduct of pharmacokinetic and toxicokinetic studies in a non-rodent species such as dogs is required per guidelines issued by International Council for Harmonisation (ICH) (ICH, 1994, 2009). The author therefore investigated accurate and reliable bioanalytical methods for the determination of ASP2151 in dog plasma and urine to help facilitate future studies.

Due to hindered rotation at the amide bond, ASP2151 has two conformers (*E* and *Z* forms, Fig. 1-7(a)(b)). The reference standard material of ASP2151 is a crystalline powder containing a single conformer (*E* form). Once the powder is dissolved in solvent, formation of the second conformer (*Z* form) of ASP2151 occurs. The two conformers of ASP2151 exhibit relatively slow inter-conversion (equilibrium time: approximately 1–8 h at 25–37°C, depending on solvent and temperature), and the conformer ratios at equilibrium depend on the solvent. ASP2151 forms two HPLC peaks under most HPLC conditions, indicating that its

conformers differ in hydrophobicity. Here, the author developed bioanalytical methods using workflows, and then validated the methods and evaluated the application of these validated methods to a pharmacokinetic study in dogs.

ASP3258 (Fig. 1-7(c)) is a phosphodiesterase 4 inhibitor for asthma (Kobayashi et al., 2012a). In two *in vivo* rat models of asthma, ASP3258 exhibited promising efficacy, with an ED_{50} of 0.81 mg/kg (Kobayashi et al., 2012a) and 0.092 mg/kg (Kobayashi et al., 2012b). These previous findings suggest that ASP3258 might be effective in the treatment of asthma.

During the drug development of ASP3258, the accurate measurement of plasma ASP3258 concentrations in rats, one of the pivotal animal disease models of asthma, is of critical importance. Given that an HPLC connected with a fluorescence detector (HPLC-FL) provided high sensitivity for ASP3258 in a preliminary experiment, the author developed an HPLC-FL method for ASP3258 in rat plasma using workflows. In addition, the author validated the method and evaluated the application of this validated method to a pharmacokinetic study in rats.

The androgen receptor inhibitor enzalutamide (formerly MDV3100; Fig. 1-7(d)) (Tran et al., 2009) has been approved in the United States, Canada, Europe, and South Korea for the treatment of metastatic castration-resistant prostate cancer (CRPC), and in Japan for CRPC patients. Further clinical development to assess the potential use of enzalutamide in treating cancers, including early-stage prostate cancer, is ongoing.

Bennett et al. developed and validated a bioanalytical method for the determination of enzalutamide and its two metabolites (inactive carboxylic acid metabolite M1 and active *N*-desmethyl enzalutamide M2, Fig. 1-7(e)(f)) in human plasma (Bennett et al., 2014). M1 and M2 were the target metabolites because they were the major drug-derived components (>10% of total exposure) in human plasma after repeated oral administration of enzalutamide at 160 mg. To comply with the ICH M3(R2) guidance (ICH, 2009), it was essential to develop

and validate a bioanalytical method for determination of enzalutamide, M1, and M2 in animal plasma in toxicology studies. In the present research, the author has developed and validated an LC-MS/MS method to measure enzalutamide, M1, and M2 in mouse plasma. In addition, the application of this validated method to a pharmacokinetic study in mice has also been described.

1.5 Aim of the present research

In the present research, following three workflows were constructed: a) workflow for the determination of the total concentration of conformers, b) workflow for the stabilization of a convertible analyte, and c) workflow for the management of interference from a convertible non-analyte.

Next, these workflows were applied to method development for ASP2151, ASP3258, and enzalutamide. Then, the validity and usefulness of the workflows were evaluated through method validation and sample analysis. Further, the pharmacokinetics of ASP2151, ASP3258, and enzalutamide were assessed using the concentration data obtained in the sample analysis.

Table 1-1. Critical issues in chromatographic bioanalytical methods during method development, method validation, and sample analysis.

Issues	References
Cross talk	(Bakhtiar and Majumdar, 2007; Unger et al., 2015)
Inadequate S/N at desired LLOQ	(Bakhtiar and Majumdar, 2007)
Variable MS response (matrix effect)	(Bakhtiar and Majumdar, 2007; Hendriks, 2009; James et al., 2004; Jemal et al., 2010; Meng et al., 2013)
Carryover	(Bakhtiar and Majumdar, 2007; Meng et al., 2013)
Visible interfering peaks in chromatograms	(Bakhtiar and Majumdar, 2007; Dadgar et al., 1995; Hendriks, 2009; Jemal and Xia, 1999)
Poor peak shape or retention of analyte	(Zhou et al., 2005)
Low or variable recovery in extraction	(Hendriks, 2009; James et al., 2004)
Variable plasma concentration due to blood-plasma partitioning	(Hilhorst et al., 2015; Unger et al., 2015)
Adsorption to sediment or analyte precipitation	(Hilhorst et al., 2015; James, 2008)
Conversion of non-analyte to analyte	(Bakhtiar and Majumdar, 2007; Dell, 2004; Hilhorst et al., 2015; James et al., 2004; Jemal et al., 2010; Li et al., 2011)
Conversion (instability) of analyte	(Briscoe and Hage, 2009; Chen and Hsieh, 2005; Hilhorst et al., 2015; Li et al., 2011; van de Merbel et al., 2014)
Interconvertible analyte	(Briscoe and Hage, 2009; Hilhorst et al., 2015; Li et al., 2011)

LLOQ; lower limit of quantification, MS; mass spectrometry, S/N; signal-to-noise ratio

Table 1-2. Typical unstable analytes and their stabilization methods.

Compound class	Stabilization method [a)]
Ester and amide	Reduced temperature, esterase inhibitor, pH (acidic) [b)]
Cytidine derivative	Cytidine deaminase inhibitor
Thiol	Derivatization
N-glucuronide	pH (acidic to neutral)
N-oxide	pH (acidic to neutral), protection from light
Phenol and alcohol	Protection from light, antioxidants, reduced temperature
Interconvertible stereoisomer	Reduced temperature, pH
Lactone and its hydroxycarboxylic acid	pH (weak acidic to neutral), reduced temperature
Photosensitive compound	Protection from light
Reactive compound	Derivatization, flash freezing
DPP-IV inhibitor	Another DPP-IV inhibitor

DPP-IV; dipeptidyl peptidase IV

a) References: (Briscoe and Hage, 2009; Chen and Hsieh, 2005; Dell, 2004; Hilhorst et al., 2015; Li et al., 2011; van de Merbel, 2013), b) If the ester is an acyl glucuronide, pH (acidic) is more effective than esterase inhibitor.

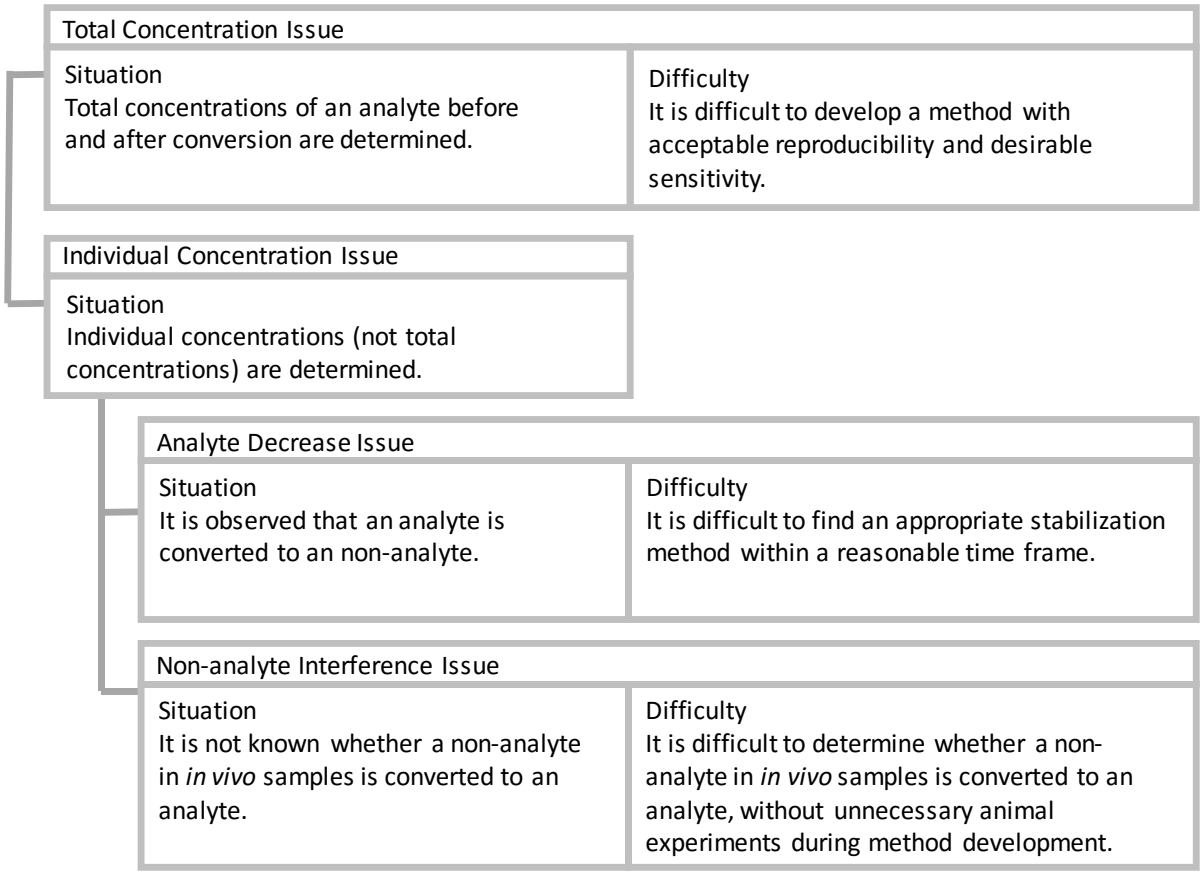

Fig. 1-1. Issues associated with conversion of analytes, dosed drugs, and their metabolites.

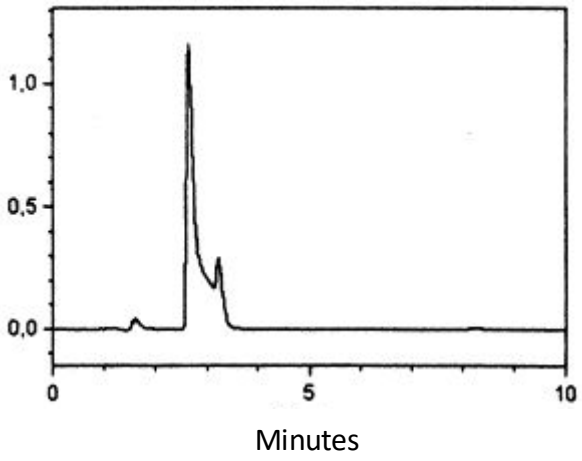

Fig. 1-2. Chromatogram of enalaprilat. Reproduced from (Bouabdallah et al., 2003) with permission from Elsevier.

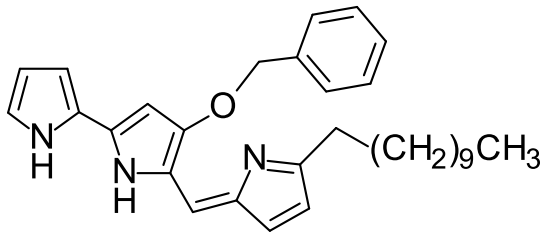

Fig. 1-3. Chemical structure of PNU-156804.

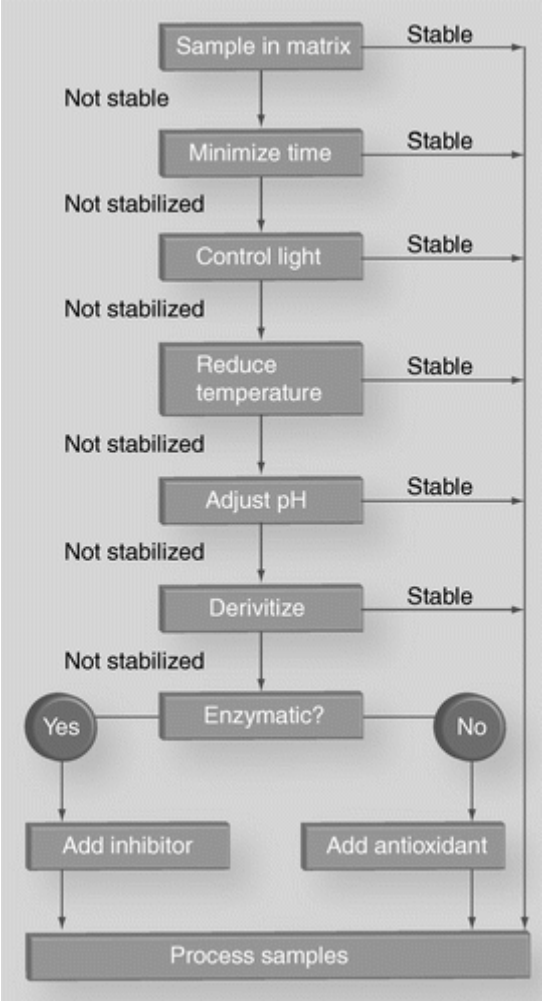

Fig. 1-4. A workflow for the stabilization of drugs in a biological matrix, proposed in (Briscoe and Hage, 2009)**.** Reproduced from the article with permission from Future Science.

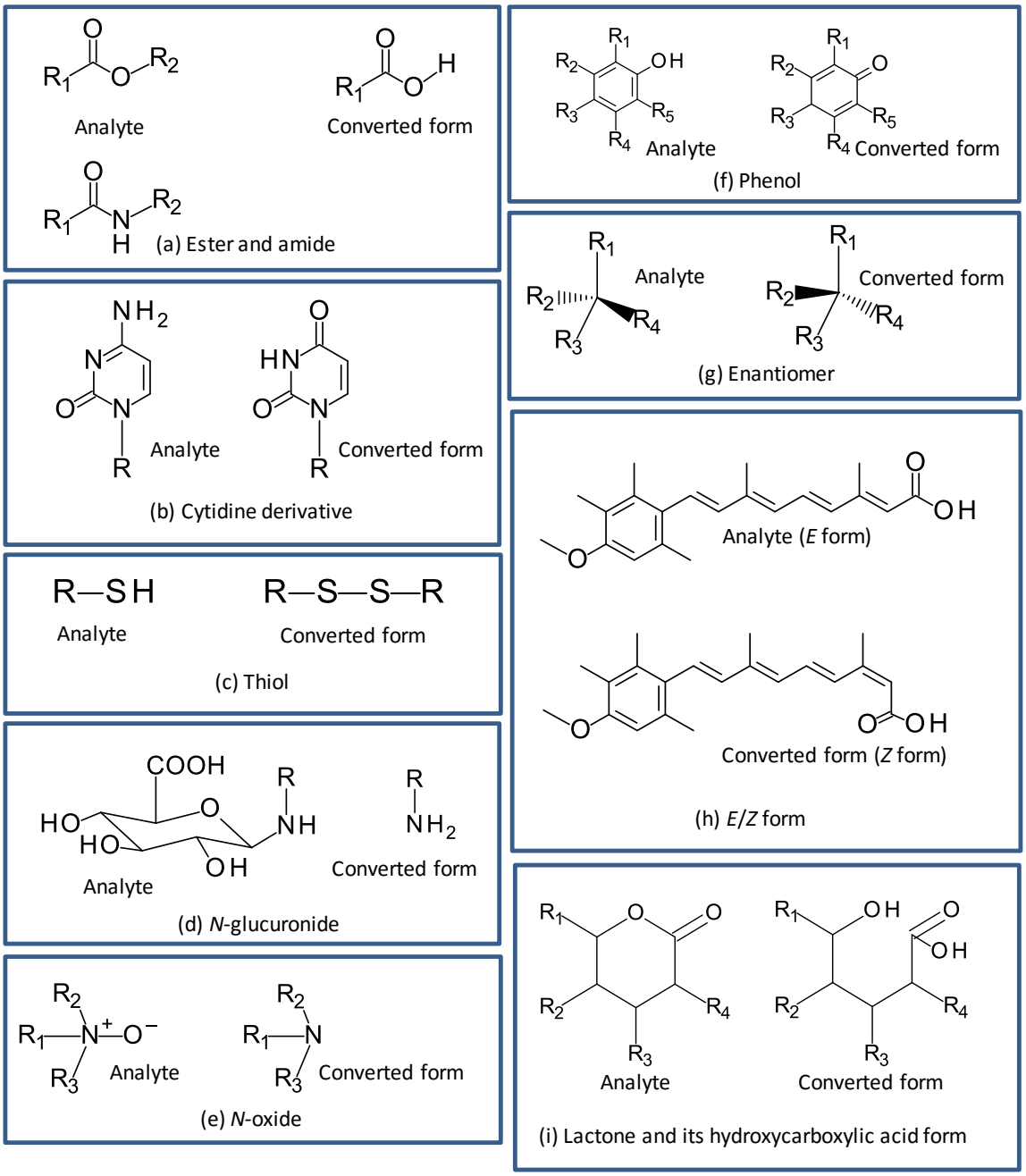

Fig. 1-5. Chemical structures of analytes and their converted forms. (a) Ester and amide, (b) cytidine derivative, (c) thiol, (d) *N*-glucuronide, (e) *N*-oxide, (f) phenol, (g) enantiomer, (h) acitretin as an example of *E*/*Z* form conversion (*E* form and *Z* form of acitretin are also known as all-*trans*-acitretin and 13-*cis*-acitretin, respectively), (i) lactone and its hydroxycarboxylic acid form.

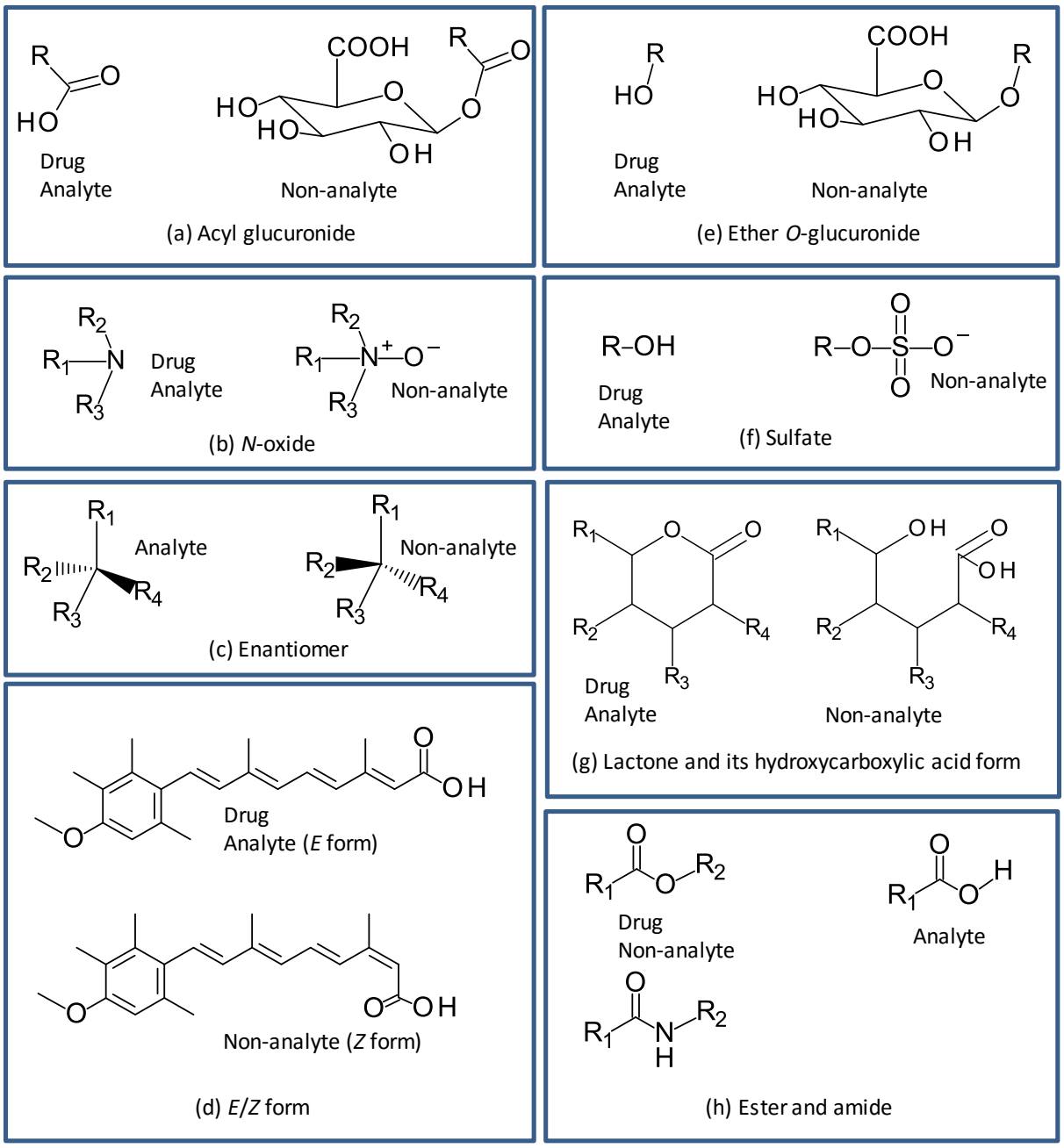

Fig. 1-6. Chemical structures of convertible non-analytes, administered drugs, and analytes. (a) Acyl glucuronide, (b) *N*-oxide, (c) enantiomer, (d) acitretin as an example of *E*/*Z* form conversion (*E* form and *Z* form of acitretin are also known as all-*trans*-acitretin and 13-*cis*-acitretin, respectively), (e) ether *O*-glucuronide, (f) sulfate, (g) lactone and its hydroxycarboxylic acid form, (h) ester and amide.

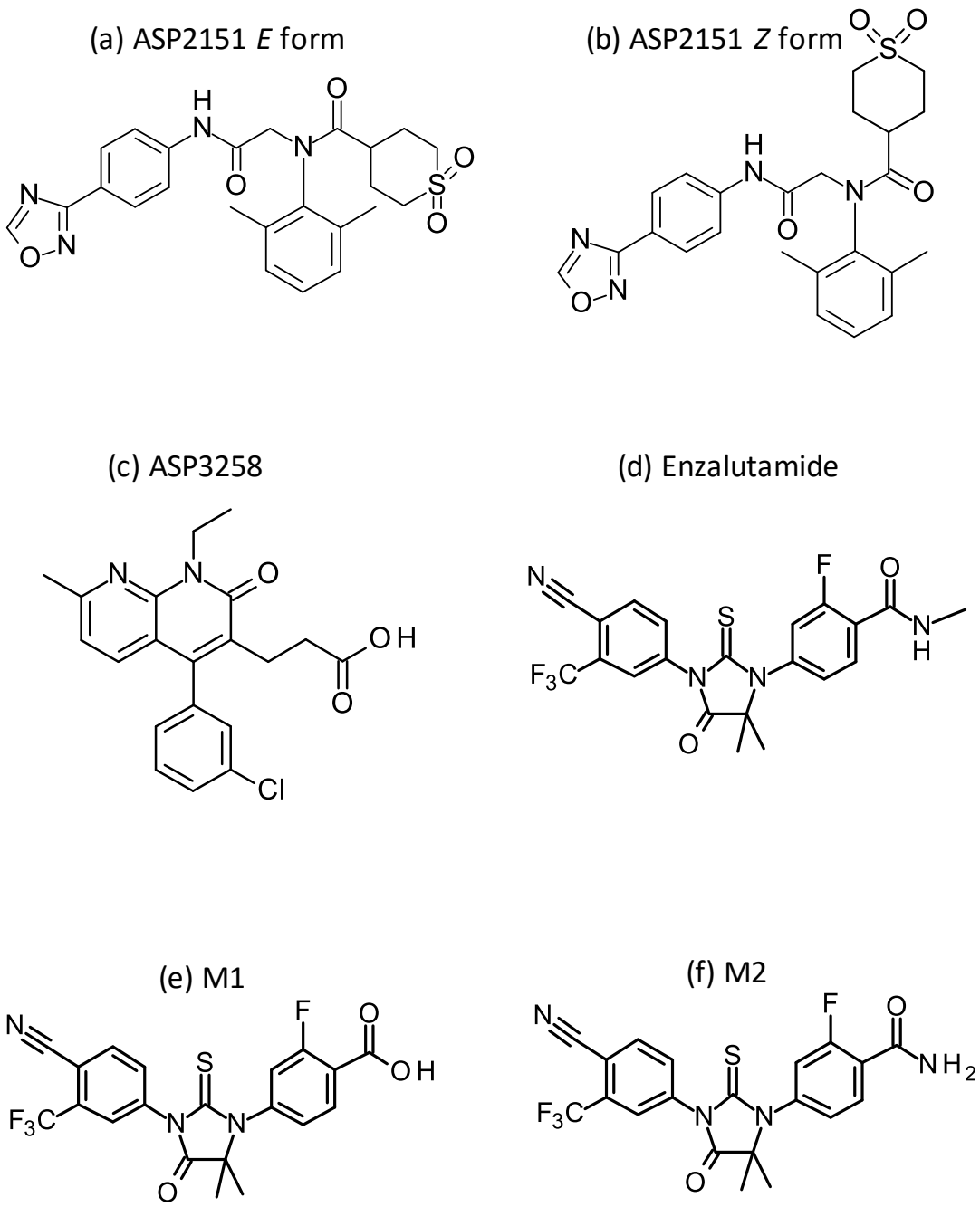

Fig. 1-7. Chemical structures of (a) ASP2151 *E* form, (b) ASP2151 *Z* form, (c) ASP3258, (d) enzalutamide, (e) enzalutamide metabolite M1, and (f) enzalutamide metabolite M2.

CHAPTER 2 DEVELOPMENT AND APPLICATION OF WORKFLOWS FOR CONVERSION ISSUES IN BIOANALYTICAL METHOD DEVELOPMENT

2.1 Introduction

Establishing an accurate and reproducible bioanalytical method plays a key role in drug development. Of many issues in bioanalysis, compound conversion may adversely and considerably exploit the time and resources required for method development, deliver inaccurate drug concentration data to pharmacologists and toxicologists, or even require medicinal chemists to seek an alternative chemical structure. When the total concentration of a convertible analyte that has conformers is measured, it is difficult to establish HPLC and pretreatment methods. Another challenge associated with compound conversion is the stabilization of convertible analytes. As there are many ways to stabilize analytes and stabilization methods depend heavily on the analyte, it is time-consuming to find the right storage condition. The final challenge the author characterizes in the present research is to identify a non-analyte that may convert to an analyte without the use of an unnecessary animal experiment.

Here, workflows for the above-mentioned issues were developed. The first workflow was to develop a determination method for the total concentration of conformers. The second workflow was to stabilize a convertible analyte. The third workflow was to manage interference from a non-analyte that may convert to an analyte.

After the development of the workflows, bioanalytical methods for ASP2151, ASP3258, enzalutamide and enzalutamides' metabolites (M1 and M2) were developed using the workflows, validated in accordance with FDA guidance (FDA, 2001), and applied to pharmacokinetic studies. The workflows were evaluated using the performance of the method in method validation and sample analysis. In addition, the measured concentration data were used for pharmacokinetic assessment of these analytes.

2.2 Materials and methods

2.2.1 Development of workflows

Based on data from the literature and the author's experiences, the following workflows were developed: a) workflow for the determination of total concentration of conformers, b) workflow for the stabilization of a convertible analyte, and c) workflow for the management of interference from a convertible non-analyte.

The literature was particularly searched for "HPLC conditions where conformers formed a single peak", "unstable analytes and their stabilization", "convertible non-analytes and their risk of interference", and "convertible non-analytes that have reference standards".

The author's experiences were drawn upon to identify available data at the initiation of method development, characterize the predictability of non-analyte formation and disposition, and estimate time for laboratory researchers to complete a certain task.

2.2.2 ASP2151

Chemicals and reagents for ASP2151

ASP2151 (international non-proprietary name, amenamevir; Fig. 2-1) and its $[CD_3]_2$-labeled analogue (AS1709867-00; IS; Fig. 2-1) were synthesized as a single conformer (*E* form) at Astellas Pharma (Tsukuba, Japan). Acetonitrile, methanol, tetrahydrofuran (THF; HPLC grade), and ammonium acetate (analytical grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ultrapure water was obtained from a Milli-Q system (EMD Millipore, Billerica, MA, USA). All other chemicals were of analytical grade.

Instruments and conditions for ASP2151

An API 4000 (SCIEX, Framingham, MA, USA) equipped with an atmospheric pressure chemical ionization (APCI) probe was used in negative multiple reaction monitoring (MRM) mode. The selected MRM detection channel was *m/z* 481→*m/z* 234 for ASP2151 and *m/z* 487→*m/z* 240 for IS. Analyst software (ver. 1.2 or 1.4.1; SCIEX) was used to control the system, collect data, and analyze mass chromatograms. When chromatographic peak parameters such as symmetry factors (S) were calculated during the development of an HPLC method, mass chromatograms were exported in comma-separated value (CSV) data format and analyzed using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

Fig. 2-2 shows a flow diagram of a column-switching HPLC system, consisting of a Shimadzu 10A series (three pumps as Pumps A, B, and C in Fig. 2-2, a degasser, an auto-injector, a column oven, and a six-port valve) (Shimadzu, Kyoto, Japan), a MAYI-ODS extraction column (4.6 mm I.D. × 10 mm L, 50 μm; Shimadzu), and a YMC-Pack Pro C18 analytical column (4.6 mm I.D. × 50 mm L, 3 μm; YMC, Kyoto, Japan) preceded by a guard column of TCI Opti-guard Fit ODS (Tokyo Chemical Industry, Tokyo, Japan). The extraction column was used at room temperature (approximately 25°C) whereas the analytical column was maintained at 40°C.

Time program and flow rates are shown in Table 2-1. After injection, the sample was chromatographed on an extraction column with a mobile phase of acetonitrile/20 mmol/L ammonium acetate (5:95, v/v) from Pump A to remove interfering endogenous components—particularly large molecules—from the biological matrix. The valve position was then changed, and the analyte and IS were back-flushed from the extraction column to an analytical column using a mobile phase of methanol/THF/20 mmol/L ammonium acetate (45:5:50, v/v/v) from Pump C. When back-flushing, auto-injector and transfer lines were washed with acetonitrile/water (50:50, v/v) from Pump B. The valve position was then

changed to the original position, and the extraction column was washed with acetonitrile/water (50:50, v/v). To re-equilibrate the extraction column to the starting condition, a mobile phase of acetonitrile/20 mmol/L ammonium acetate (5:95, v/v) was delivered to the extraction column. Total run time was 10 min.

Calibration samples for ASP2151

Stock solutions of ASP2151 (1 mg/mL) and IS (0.5 mg/mL) were prepared by dissolving compounds in acetonitrile/water (50:50, v/v), and solutions were stored at 4°C. Working solutions (final acetonitrile concentration: 20% [v/v]) were prepared by diluting stock solutions with water and acetonitrile. For plasma, calibration standard samples were prepared by spiking working solutions in blank dog plasma at final plasma concentrations of 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL. For urine, calibration standard samples were prepared by spiking the working solutions in acetonitrile-added blank dog urine (acetonitrile:dog urine=99:1) at final urine concentrations of 0.5, 1, 2, 5, 10, 20, 50, and 100 µg/mL.

Quality control samples for ASP2151

For plasma, stock solutions of ASP2151 (1 mg/mL) were prepared by dissolving the compound in acetonitrile/water (50:50, v/v) and stored at 4°C. Working solutions were prepared by diluting the stock solutions with acetonitrile/water (50:50, v/v). Quality control (QC) samples were prepared at low (15 ng/mL), medium (75 ng/mL), and high (750 ng/mL) concentrations in dog plasma by 100-fold dilution of the working solution. For urine, stock solutions of ASP2151 (7.5 mg/mL) were prepared by dissolving the compound in acetonitrile and stored at 4°C. Working solutions were prepared by diluting the stock solutions with acetonitrile. QC samples were prepared at low (1.5 µg/mL), medium (7.5 µg/mL), and high

(75 μg/mL) concentrations in dog urine by 100-fold dilution of the working solution.

Sample preparation for ASP2151

Study plasma samples or QC plasma samples (0.1 mL) were mixed with acetonitrile/water (20:80, v/v) (0.05 mL) and IS working solution (250 ng/mL, 0.05 mL). For calibration standard plasma samples, blank plasma (0.1 mL) was mixed with working solutions (0.05 mL) and IS working solution (0.05 mL) to ensure that the components of the calibration standard plasma samples were the same as study plasma samples. The resultant samples were filtered with a 96-well filter plate (3M, St. Paul, MN, USA) by sucking. Following filtration, a 0.05 mL aliquot of the supernatant was injected into the column-switching HPLC system. Urine samples were diluted 100-fold with acetonitrile, and 0.1 mL aliquots of the diluted samples were processed in the same manner as plasma samples.

Method validation for ASP2151

Method validation was conducted in accordance with FDA guidance (FDA, 2001). The selectivity of the method was assessed by ensuring no interference of the analyte and IS in the mass chromatograms of blank individual samples from six dogs. The LLOQ was defined as the lowest concentration at which the accuracy was within ±20% and the precision ≤20%. The LLOQ was evaluated by analyzing samples in six replicates. Accuracy was expressed as relative error (RE) and calculated using the following equation:

RE (%) = (mean measured concentration – nominal concentration)/(nominal concentration) × 100

Precision was expressed as a coefficient of variation (CV) and calculated using the following equation:

CV (%) = standard deviation of measured concentrations/

mean measured concentration × 100

Calibration curves were constructed by analyzing spiked calibration samples. Peak area ratios of ASP2151 to IS were plotted against analyte concentrations, and the curves were fitted to the equations via linear regression with a weighting factor (i.e. $1/x^2$) in the concentration range of 5 to 1000 ng/mL for plasma and 0.5 to 100 μg/mL for urine.

The accuracy and precision were determined by analyzing low, medium, and high QC samples. To determine intra- and inter-day accuracy and precision, six replicate QC samples at each concentration were analyzed over three days. At these three concentration levels, accuracy within ±15% and precision ≤15% was required.

The stability of ASP2151 in dog plasma and urine under various storage or handling conditions was examined by analyzing three replicate low and high QC samples at each concentration and comparing the measured concentrations with nominal concentrations. Short-term stability was determined after exposure of the QC samples at room temperature for 24 h. Long-term stability was assessed after storage of the QC samples at −20°C for 183 days for plasma and 82 days for urine. Freeze-thaw stability was determined after three freeze-thaw cycles of the QC samples. The post-preparative stability of ASP2151 was also evaluated.

All stability data were expressed as Stability (%) and calculated using the following equation:

Stability (%) = (measured concentration – nominal concentration)/(nominal concentration) × 100

Stability within ±15% was required.

Pharmacokinetic study of ASP2151

To assess the application of the validated method to pharmacokinetic study, the oral

and intravenous (i.v.) pharmacokinetic profile of ASP2151 in dogs was determined. Beagle dogs from Kitayama Labes (Iwakuni, Japan) were allowed free access to water. Four animals were fasted overnight before drug administration, after which they were allowed free access to food from 6 h after dosing. Following a single oral administration of ASP2151 at doses of 0.3, 1, and 3 mg/kg, blood samples were collected at pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8, 12, and 24 h post-dose. Following a single i.v. administration of ASP2151 at a dose of 1 mg/kg, blood samples were collected at 0.1 h post-dose in addition to the same sampling time points as oral administration. Blood samples were collected using syringes containing sodium heparin from the external jugular vein of animals. All blood samples were centrifuged immediately after collection to obtain plasma. In parallel with blood collection after a single i.v. administration, urine samples were collected at five intervals (0–4 h, 4–8 h, 8–12 h, 12–24 h, and 24–36 h post-dose) by placing animals in metabolic cages and the total urine volumes during the sampling period were recorded. To ensure uniformity, urine samples were vigorously mixed for 5 min using a magnetic stirrer, and approximately 1 mL aliquots were taken for bioanalysis. Plasma and urine samples were stored at −20°C until analysis. Plasma and urine concentration-time data were analyzed using Pharsight WinNonlin version 4.0 (Mountain View, CA, USA). This animal study was approved by the Institutional Animal Care and Use Committee of the test facility and was performed in accordance with the ethics criteria contained in the bylaws of the committee.

2.2.3 ASP3258

Chemicals and reagents for ASP3258 and ASP3258-AG

ASP3258 and its IS (AS1406604-00) were synthesized at Astellas Pharma (Fig. 2-3). Acetonitrile and ethyl acetate (HPLC grade) were purchased from Kanto Chemical (Tokyo, Japan), while acetone (HPLC grade) was purchased from Wako Pure Chemical. Ultrapure

water was obtained from a Milli-Q system (EMD Millipore). All other chemicals and solvents were of analytical grade.

Instruments and conditions for ASP3258 and ASP3258-AG

All analyses were performed using a Shimadzu 10A series HPLC system coupled with a Shimadzu RF-10AXL FL detector. The mobile phase consisted of acetonitrile/0.5% acetic acid (50:50, v/v). Analytes were detected using wavelengths of 315 nm for excitation and 365 nm for emission. Data were collected using Millennium32 chromatography manager version 3.06 (Waters, Milford, MA, USA).

To quantify ASP3258, chromatography was performed on a Shiseido Capcell Pak C18 UG120 column (3.0 mm I.D. × 150 mm L, 5 μm) (Tokyo, Japan), preceded by a Shiseido Capcell Pak C18 UG120 guard column (2.0 mm I.D. × 10 mm L, 5 μm) at 45°C. The flow rate was 0.4 mL/min.

To detect ASP3258-AG, chromatography was performed on a Shiseido Capcell Pak C18 UG120 column (4.6 mm I.D. × 250 mm L, 5 μm) at 40°C, without a guard column. The flow rate was 1.0 mL/min.

Calibration samples and quality control samples for ASP3258

Stock solutions of ASP3258 (0.2 mg/mL) and AS1406604-00 (IS, 0.2 mg/mL) were prepared by dissolving the compounds in ethanol. Working solutions were prepared by diluting the stock solutions with ethanol. Calibration standard samples were prepared by spiking working solutions in blank rat plasma at final concentrations of 2.5, 5, 10, 25, 50, 100, and 250 ng/mL. QC samples at low, medium, and high concentrations were prepared at concentrations of 7.5, 80, and 200 ng/mL in rat plasma.

Sample preparation for ASP3258

Extraction of ASP3258 was carried out by SPE. Plasma samples (0.2 mL) were mixed with 50 mmol/L phosphoric acid (3 mL), ethanol (0.1 mL), and IS working solution (400 ng/mL, 0.1 mL) and loaded onto ISOLUTE C18 (EC) SPE cartridges (500 mg/3 mL; International Sorbent Technology, Hengoed, United Kingdom), which were sequentially pre-conditioned with 3 mL of acetone, water, and 50 mmol/L phosphoric acid. Loaded cartridges were washed with 3 mL of 50 mmol/L phosphoric acid and 3 mL of a mixture of 50 mmol/L phosphoric acid/acetone (55:45, v/v). To the washed cartridges, 3 mL of ethyl acetate was added. Eluent was evaporated to dryness at 35°C, and residue was reconstituted in 0.2 mL of acetonitrile/0.5% acetic acid (30:70, v/v). Following centrifugation (900×*g*, ambient temperature, 5 min), a 0.05 to 0.08 mL aliquot of supernatant was injected into the HPLC-FL.

Detection of ASP3258-AG in *in vivo* samples

Two extraction methods were utilized to detect ASP3258-AG and its isomers comprehensively. One extraction method was LLE, which efficiently extracts ASP3258-AG and its isomers at retention times of 6.1 min and 7.9 min. Plasma samples (0.5 mL) were mixed with 500 mmol/L phosphoric acid (2 mL). After extraction with chloroform (5 mL) for 10 min and centrifugation, the chloroform layer was transferred to a new test tube. The extract was evaporated to dryness at 35°C, and the residue was reconstituted in 0.2 mL of acetonitrile/0.5% acetic acid (50:50, v/v). A 0.05 mL aliquot of supernatant was injected into the HPLC-FL.

The second extraction method was SPE, which efficiently extracts ASP3258-AG and its isomers at a retention time of 13.5 min. Plasma samples (0.5 mL) were mixed with 50 mmol/L phosphoric acid (3 mL) and loaded onto OASIS HLB SPE cartridges (60 mg;

Waters), which were sequentially pre-conditioned with 3 mL of acetone, water, and 50 mmol/L phosphoric acid. Loaded cartridges were washed with 3 mL of 50 mmol/L phosphoric acid and 3 mL of a mixture of 50 mmol/L phosphoric acid/acetone (60:40, v/v). To the washed cartridges, 1 mL of acetone and 2 mL of ethyl acetate/acetone (80:20, v/v) was added sequentially. Eluent was evaporated to dryness at 35°C, and residue was reconstituted in 0.2 mL of acetonitrile/0.5% acetic acid (50:50, v/v). A 0.05 mL aliquot of supernatant was injected into the HPLC-FL.

Twelve-week-old male Fischer rats obtained from Charles River Laboratories Japan (Atsugi, Japan) were allowed free access to water and food. Following a single oral administration of ASP3258 at a dose of 1 mg/kg, blood samples were collected at 0.5 h post-dose. Blood samples were collected using syringes containing heparin from the inferior vena cava of three animals or hepatic vein of three animals under ether anesthesia, after which animals were sacrificed *via* exsanguination. Plasma samples were obtained by centrifugation (3400×*g*, 4°C, 3 min), immediately acidified (<5 min between blood sampling and acidification), and analyzed. This animal study was approved by the Institutional Animal Care and Use Committee of the test facility and was performed in accordance with the ethics criteria contained in the bylaws of the committee.

Method validation for ASP3258

Selectivity, sensitivity, linearity, accuracy, precision, extraction recovery, and stability were examined and acceptance criteria were predefined in accordance with FDA guidance (FDA, 2001).

Pharmacokinetic study of ASP3258

Six-week-old male Fischer rats obtained from Charles River Laboratories Japan

(Yokohama, Japan) were allowed free access to water and acclimated for a week. Animals were fasted overnight before drug administration, after which they were allowed free access to food from 4 h after dosing. Following a single i.v. administration of ASP3258 at a dose of 0.1 mg/kg, blood samples were collected at 0.1, 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 24 h post-dose. Blood samples were collected using syringes containing sodium heparin from the inferior vena cava of three animals per time point under ether anesthesia, after which animals were sacrificed *via* exsanguination. Plasma samples were obtained by centrifugation (1800×*g*, ambient temperature, 15 min) and stored at −20°C until analysis. Mean plasma concentration-time data were analyzed using Pharsight WinNonlin version 3.1. This animal study was approved by the Institutional Animal Care and Use Committee of the test facility and was performed in accordance with the ethics criteria contained in the bylaws of the committee.

2.2.4 Enzalutamide

Chemicals and reagents for enzalutamide and its metabolites

Enzalutamide, M1, and M2 (Fig. 2-4) were synthesized at Albany Molecular Research (Albany, NY, USA). $^{13}CD_3$-labeled analogues (Fig. 2-4) were synthesized at J-Star Research (South Plainfield, NJ, USA). $[CD_3]_2$-labeled analogues (Fig. 2-4) were synthesized at PerkinElmer (Boston, MA, USA). Acetonitrile, methanol, and *tert*-butyl methyl ether (TBME) (HPLC grade) were purchased from Wako Pure Chemical. 2,2-Dichlorovinyl dimethyl phosphate (DDVP) was of residual pesticide test grade and was purchased from Wako Pure Chemical. Ultrapure water was obtained from a Milli-Q system (EMD Millipore). All other chemicals were of analytical grade.

Instruments and conditions for enzalutamide and its metabolites

A Shiseido Nanospace SI-2 HPLC system, consisting of two pumps, a degasser, an auto-injector, and a column oven, was used for sample injection and separation. Chromatography was performed on a Cadenza CD-C18 column (2.0 mm I.D. × 30 mm L, 3 μm; Imtakt, Kyoto, Japan) at 40°C. A solvent gradient was formed from 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B), at a flow rate of 0.3 mL/min with the following characteristics: solvent B concentration was increased from 50% to 80% from 0 to 1.5 min and maintained at 80% from 1.5 to 2.0 min, then at 95% from 2.0 to 6.5 min. Solvent B concentration was then cycled back to 50% (initial concentration of solvent B) for column re-equilibration.

An API 4000 (SCIEX) was used in positive electrospray ionization (ESI) mode set at a temperature of 500°C. The MRM detection channels for enzalutamide, M1, and M2 were *m/z* 465.2 → *m/z* 209.1, *m/z* 452.2 → *m/z* 196.1, and *m/z* 451.2 → *m/z* 195.1, respectively. The MRM detection channels for $^{13}CD_3$-enzalutamide, $^{13}CD_3$-M1, and $^{13}CD_3$-M2 were *m/z* 469.2 → *m/z* 213.1, *m/z* 456.2 → *m/z* 200.2, and *m/z* 455.2 → *m/z* 199.2, respectively. When $[CD_3]_2$-labeled analogues were used as IS in place of $^{13}CD_3$-labeled analogues, the MRM detection channels for $[CD_3]_2$-enzalutamide, $[CD_3]_2$-M1, and $[CD_3]_2$-M2 were *m/z* 471.2 → *m/z* 215.1, *m/z* 458.2 → *m/z* 202.2, and *m/z* 457.2 → *m/z* 201.2, respectively. Analyst software (ver. 1.4 or 1.5; SCIEX) was used to control the system, collect data, and analyze mass chromatograms.

Calibration samples and quality control samples for enzalutamide and its metabolites

Stock solutions of enzalutamide and M2 (5 mg/mL) and their respective analogues (IS, 0.1 mg/mL) were prepared by dissolving the compounds in acetonitrile, and stored at 4°C. Stock solutions of M1 (5 mg/mL) and its analogue (IS, 0.1 mg/mL) were prepared by

dissolving compounds in dimethyl sulfoxide (DMSO) and stored at 4°C. Working solutions (final DMSO concentration: 20% [v/v]) were prepared by diluting stock solutions with DMSO and acetonitrile. Calibration samples were prepared by spiking the working solutions in blank matrix at final plasma concentrations of 20, 50, 100, 500, 1000, 2000, 5000, 10000, and 50000 ng/mL. QC samples at LLOQ, low, medium, and high concentrations were prepared at 20, 60, 1000, and 40000 ng/mL, respectively.

Sample preparation for enzalutamide and its metabolites

Plasma samples were mixed with 2% DDVP methanol solution at a volumetric ratio of 99:1 to prevent enzymatic conversion of M2 to M1. These samples (0.05 mL) were then mixed with IS working solution (200 ng/mL each for $^{13}CD_3$-labeled IS or 2 μg/mL each for $[CD_3]_2$-labeled IS, 0.025 mL) and 0.1% formic acid in water (0.2 mL). The mixture was vortexed with TBME (1 mL) for 10 min and centrifuged (10000×*g*, ambient temperature, 3 min). A 0.2 mL aliquot of the organic layer was transferred to a clean test tube and evaporated under nitrogen at 40°C. The residue was reconstituted with 0.25 mL of 0.1% formic acid in methanol/water (40:60, v/v). A 0.005 mL aliquot of the reconstituted sample was then injected into the LC-MS/MS system.

Method validation for enzalutamide and its metabolites

Selectivity, sensitivity, linearity, accuracy, precision, and stability were examined and acceptance criteria were predefined in accordance with FDA guidance (FDA, 2001).

Method validation was first conducted using $^{13}CD_3$-labeled analogues as IS. Due to the availability of the other set of IS, partial validation was then performed using $[CD_3]_2$-labeled IS.

Pharmacokinetic study of enzalutamide

The validated method using $[CD_3]_2$-labeled IS was used in a pharmacokinetic study in mice to measure the concentrations of enzalutamide, M1, and M2.

To determine the plasma pharmacokinetic profile following multiple oral doses of enzalutamide, blood samples were drawn from the inferior vena cava of mice (Crlj:CD1[ICR], Charles River Japan) of both sexes. At the initiation of dosing, mice were 7 weeks old. Animals were allowed free access to water and food. Enzalutamide was administered once daily for four weeks at 10, 30, and 60 mg/kg/day to mice using flexible stomach tubes. Blood samples were collected on Day 1 and the final day of administration using syringes containing dipotassium ethylenediaminetetraacetic acid, and mixed with 2% DDVP methanol solution at a volumetric ratio of approximately 99:1 in centrifuge tubes standing in an ice-water bath. Samples were then centrifuged to obtain plasma and stored at −20°C until analysis. Plasma concentration-time data were analyzed using Pharsight WinNonlin version 5.2.1.

This animal study was approved by the Institutional Animal Care and Use Committee of the test facility and was performed in accordance with the ethics criteria contained in the bylaws of the committee.

2.3 Results and discussion

In this thesis, most of the pre-validation results have been omitted as they overlap with the validation study results. If the pre-validation results did not meet acceptance criteria and the method was modified, the results and the method modifications are presented.

2.3.1 Identification of key tasks and establishment of workflows

Determination of total concentration of conformers

The goal of this workflow is to develop a method to determine the total

concentration of conformers. Therefore, the first task of this workflow was identified to be confirmation whether the analyte has conformers and whether total concentrations of the conformers are to be measured (Fig. 2-5). In other words, the first task is to confirm whether the analyte and method are within the scope of this workflow. Confirmation should be completed by consulting with scientists involved in the physicochemistry, pharmacology, and toxicology of the drug.

As the next necessary task, the establishment of a detection method was identified. Establishment of the detection method makes it possible to monitor the elution of an analyte by HPLC and examine the HPLC method in a subsequent step.

Establishment of an HPLC method was then identified as the next task. An extensive literature search revealed that a high column temperature was most frequently used to obtain a single HPLC peak. It was considered to be rational that many conformers would form a single peak with higher column temperature, as interconversion generally becomes faster at higher temperatures. In addition, there was one case study in which a single HPLC peak was observed by changing the mobile phase pH. As it is known that the mobile phase pH affects ionization of the analyte as well as ionization of silanols on the HPLC stationary phase, it was deemed generally likely that the pH of the mobile phase has an influence on the HPLC separation of conformers. In addition, the author judged that column temperature and mobile phase pH were easy to examine during the course of method development and feasible to repeat in sample analysis. Taken together, column temperature and mobile phase pH were placed in this order as steps in the workflow.

While all the publicly available case studies were successful using either column temperature or mobile phase pH, it was considered prudent to assume a case in which neither column temperature nor mobile phase pH provides a single HPLC peak. Besides, column temperature and mobile phase pH are only part of the HPLC condition. While a single peak

can be obtained by decreasing theoretical plate number (*N*), retention factor (*k*), and separation factor (α), the two formers generally deteriorates the separation between the analyte and endogenous components and therefore adversely affects the accuracy and precision of the method. Given that, the author decided to focus on α. In analysis of general compounds, other elements in HPLC conditions are known to effectively change HPLC separation. Thus, it was deemed reasonable to continue to search for appropriate HPLC conditions when experiments using higher column temperature and mobile phase pH change were unsuccessful. The organic modifier of the mobile phase and column chemistry are often useful to change α. While column chemistry has a wide range of options, the organic modifier of the mobile phase has only three options (e.g. acetonitrile, methanol, and THF) in reality. Thus, the author found that the latter would require less time to complete the experiment than the former. In addition, the latter was considered more advantageous from the viewpoint of expense. Taken together, it was decided to place organic modifier and column chemistry in this order in the workflow. While the addition of the ion pair reagent is known to be useful for a change in α, this was excluded from the workflow as the ion pair reagents cause the suppression of ionization in LC-MS/MS.

The task of development of a pretreatment method was included in this workflow, as there are a number of considerations in the pretreatment method for conformers. Compared with protein precipitation, LLE, SPE, and column-switching are better methodologies in terms of extraction selectivity and ion suppression/enhancement in LC-MS/MS. Of these three methodologies, column-switching was found to be an easy way to reduce the influence of interconversion during pretreatment, because 1) it allows easy control of the influence of light and temperature, 2) all samples are processed in the same time duration, and 3) it requires only a short time. In addition, if the conformers exhibit differences in hydrophobicity, the issues with hydrophobicity can be managed using extraction columns with an aqueous mobile

phase in which both conformers are retained at the entrance of the extraction columns. Further, column-switching was considered to be easily examined in method development and feasible to repeat in sample analysis. Taken together, the author decided to recommend column-switching as pretreatment.

The order between HPLC method examination and pretreatment examination was decided as follows. Generally, bioanalytical methods are developed in this order. In addition, HPLC condition plays an important role in the column-switching method, in comparison with the other pretreatment methods, and thus facilitates the establishment of the column-switching method. Based on these background factors, HPLC method examination and pretreatment examination were placed in this order in the workflow.

The constructed workflow is called the “total concentration workflow” hereafter. It can be abbreviated to “total conc. workflow” in figures.

The total concentration workflow is expected to serve to improve efficiency in method development. This improvement is characterized as follows. For the HPLC method, the workflow eliminates ineffective examinations (such as flow rates, buffer components, buffer concentrations, gradient programs, or injection solutions) and time-consuming examinations (such as combination of column chemistry and mobile phase). For the pretreatment method, the workflow eliminates less optimal methodologies (SPE and LLE etc.). The elimination of these unnecessary experiments is considered to save considerable time and energy in method development for total concentration of conformers.

Stabilization of convertible analyte

The goal of this workflow is stabilization of an analyte. Accordingly, the first task of this workflow was identified to be judgment of the need for stabilization (Fig. 2-6). In practice, a known quantity of the analyte should be spiked in blank matrix during

pre-validation. The samples should be stored for a certain time and quantified. The difference between the nominal and measured concentrations should be calculated to evaluate a concentration change of the analyte.

If a decrease in concentration is not observed, no stabilization measure was considered needed (e.g. end of the workflow). If a decrease in concentration is detected, analyte conversion will be suspected. A concentration decrease does not always mean analyte conversion. If the matrix is urine, a decrease can be observed for the following reasons: (i) adsorption of the analyte to a container (Li et al., 2011), (ii) precipitation of the analyte (James, 2008), and (iii) co-precipitation of the analyte with endogenous components (Hilhorst et al., 2015). Therefore, to exclude these factors, the author recommended that the concentration change in matrix be examined after adding organic solvent or surfactant to the matrix (this examination was not included in Fig. 2-6, as this issue was considered exceptional). If the concentration change persists, the concentration change must be due to analyte conversion (instability) and it was then considered necessary to take actions to stabilize the analyte.

The next task identified was to examine whether the analyte falls into a typical unstable compound class (Fig. 2-7) using chemical structure, physicochemical data (photosensitivity and interconvertible stereoisomers), and MOA. Available drug metabolism data for the analyte were judged sometimes helpful for this process. For example, if the drug metabolism study identifies a metabolite in which the ester moiety of the analyte is hydrolyzed, the instability may be ascribable to the degradation by hydrolysis of the ester moiety. In addition, pH and metabolizing enzyme disposition in the matrix were deemed useful in speculating a cause of the instability.

If the analyte falls into one of the compound classes listed in Fig. 2-7, the next task identified was to experimentally test the recommended stabilization measures. In addition, it

was judged important to prepared suggestions for the case in which the analyte is not stabilized using the recommended stabilization measures or does not fall into any of the compound classes listed in Fig. 2-7. Thus, stabilization using Briscoe's workflow was placed as the next task (Fig. 1-4 and Fig. 2-6).

The constructed workflow is called the "analyte stabilization workflow" hereafter. It can be abbreviated to "stabilization workflow" in figures.

The analyte stabilization workflow is expected to serve to improve efficiency in the development of stabilization methods. It is expected that a laboratory researcher can stabilize the analyte in fewer steps using this workflow than using Briscoe's, provided that the analyte falls into one of the typical unstable compound classes. For example, if the analyte is ester and esterase inhibitor is necessary to stabilize the analyte, it is estimated that Briscoe's workflow would require seven steps, whereas the new workflow would need only two steps.

Management of interference from a non-analyte that may convert to an analyte

In order to manage interference from a non-analyte that may convert to an analyte without exploiting resources, an identified task was to estimate whether an interfering compound is likely to be present in *in vivo* samples, and if so, what is its compound class (Fig. 2-8). For this task, the following information should be used:

- Chemical structures of analytes
- Whether chemical structures of dosed drugs and precursor metabolites suggest their conversion to analytes (Typical chemical structures of the non-analyte are esters and amides. It was judged prudent to consult with the analyte stabilization workflow and examine whether the non-analyte can be readily changed into the analyte due to the typical causes of instability.)
- Whether interconvertible stereoisomers can be produced (physicochemical and drug

metabolism data)

The risk of interference was considered to vary depending on the compound class of the non-analyte. For example, while the formation of *N*-oxide is suggested by the chemical structures of numerous analytes, only a few of the analytes actually have *N*-oxide in *in vivo* samples, based on the author's experiences. In addition, almost all ether *O*-glucuronides do not interfere with the quantification of their aglycones, as ether *O*-glucuronides are generally stable. Some compound classes are always accompanied by reference standards. Considering these characteristics, potential interfering compounds were divided into four categories (Fig. 2-8). *N*-glucuronides were excluded from this, as these are less likely to be formed in animals and the present research focuses on animals.

As the aim of this workflow is to considerably reduce the possibility of interference using reasonable resources, it was judged acceptable that there is a very limited possibility (i.e. exceptional cases that current science cannot exclude efficiently) that an interfering compound would be found in the first sample analysis study and that method development and validation would need to be conducted again.

If the analyte does not fall into either of the categories, no further task was deemed necessary (e.g. the end of this workflow) as the author concluded that the risk of interference by convertible non-analyte is small enough to accept. If the analyte falls into one of the categories, the next task identified was to take any actions necessary to mitigate the risk. Carboxylic acid compounds fell into the high-risk category (category 1) as AGs are often present in *in vivo* samples and are generally unstable. Therefore, it was recommended to experimentally examine the presence of AG in *in vivo* samples (Fig. 2-9). If AG is observed, the next task was to take care of the AG during sample collection, storage, and analysis. If AG is not observed, no further task was deemed necessary (e.g. the end of this workflow).

Tertiary amines and interconvertible stereoisomers fell into the medium-risk

category (category 2) as *N*-oxides and interconvertible stereoisomers are less likely to be present in *in vivo* samples and are generally unstable. An identified task was to consult the *in vitro* drug metabolism data to determine the presence of non-analytes (Fig. 2-10). *In vitro* drug metabolism data are usually available when a laboratory researcher plans to analyze samples in accordance with the FDA guidance. If not available, the author recommended to conduct an *in vitro* metabolism study. If the non-analyte is not produced *in vitro*, no further task was deemed necessary (e.g. the end of this workflow). If it is produced *in vitro*, the next task was determined to be an experiment concerning the presence of the non-analyte *in vivo*. If the non-analyte is observed, the suggestion was to take care of the non-analyte during sample collection, storage, and analysis. If it is not observed, no further task was deemed necessary (e.g. the end of this workflow).

Alcohols, phenols, primary amines, and secondary amines (including hydroxyamines) fell into the low-risk category (category 3) as ether *O*-glucuronides and sulfates are generally stable. It was considered reasonable to wait for the reference standard or *in vivo* samples (Fig. 2-11). When these are available, it was recommended to examine the stability of the non-analyte in biological matrix experimentally before a number of study samples are measured. If conversion of the non-analyte to the analyte is observed, the next action identified was to take care of the non-analyte during sample collection, storage, and analysis.

Lactones and their hydroxycarboxylic acid forms fell into category 4. If the chemical structure of a dosed drug or precursor metabolite suggests their degradation to an analyte, they should also fall into this category. With regard to non-analytes in this category, reference standards are usually available. One task identified was to spike the reference standard of the non-analyte in blank biological matrix and check the conversion (Fig. 2-12). If conversion is observed, the suggestion was to take care of the non-analyte during sample

collection, storage, and analysis. If conversion is not observed, no further task was deemed necessary (e.g. the end of this workflow).

The workflow includes suggestions to examine the presence of the convertible non-analytes *in vitro* or *in vivo*. To ensure these tests are conducted properly and the workflow works well, some points to consider were identified in this research, as described below. It was considered important to avoid the conversion of non-analytes (for example, handle samples at reduced temperature) and analyze the samples using the analytical instrument immediately. For stereoisomers, an appropriate HPLC method should be available to separate the isomers. In any case, if a quantitative or qualitative method is available for the non-analyte, it was recommended to use the method. If no analytical method for the non-analyte is available, it might be advisable to use HPLC-mass spectrometry and examine whether an ion derived from the non-analyte is detected.

When a non-analyte is observed *in vivo*, the workflow requires stabilization of the non-analyte. The author considered that this task should be supported by the analyte stabilization workflow. In addition, to remove the influence of the degradation of the non-analyte in an early phase of pretreatment, it was considered worth examining whether an extraction procedure can selectively extract the analyte and discard the non-analyte.

While this workflow deals with the case in which a non-analyte interferes with analyte quantification, this workflow is also applicable to multi-analyte methods in which one of the analytes interferes with quantification of another analyte. In these cases, the interfering analyte is known to be present in *in vivo* samples and a reference standard for the interfering analyte must be available. The author recommended experiments on conversion of the interfering analyte during stability tests in pre-validation.

The constructed workflow is called the “interference management workflow” hereafter. It can be abbreviated to “interference workflow” in figures.

The interference management workflow is expected to manage the interference risk without requiring much in the way of resources. The expected improvement is as follows. Unlike the previous study, the interference management workflow does not always require extensive *in vivo* experiments. This new workflow is expected to eliminate many extensive *in vivo* experiments without leading to frequent issues from non-analyte conversion. Unlike current industry practice, the interference management workflow does not always skip an *in vivo* experiment. According to this new workflow, analytes with high risk, which represent only a small percentage of the whole picture, would require a slightly longer period for method development than the current industry practice. However, this workflow is expected to considerably reduce the possibility that an interfering non-analyte would be found in sample analysis, and thus substantially avoid the inconvenience of having laboratory researchers spend several months to conduct method development and validation again, and provide correct concentration data of the study samples.

2.3.2 Application to ASP2151

Method development for ASP2151

An overview of method development for ASP2151 is shown in Fig. 2-13. First, potential conversion of non-analyte to analyte was examined according to the interference management workflow. While an oxadiazole ring of ASP2151 has a tertiary amine, it was judged that this is not metabolized to *N*-oxide, considering examples of similar compounds. In conclusion, ASP2151 did not fall into either of the four categories shown in the workflow and it was therefore judged unlikely that conversion of non-analyte could interfere with ASP2151 quantification.

The author then examined whether the ASP2151 method is within the scope of the total concentration workflow. ASP2151 has interconvertible conformers. In addition, the drug

developer of ASP2151 decided that the total concentration of the conformers should be assessed in the determination of ASP2151 in biological fluids. Based on this information, the ASP2151 method was judged to be within the scope of the total concentration workflow.

Following the total concentration workflow, a detection method using mass spectrometry was then determined as follows. ESI and APCI in both polarities were tested. Based on the intensity of protonated or deprotonated molecular ions of ASP2151, the APCI negative mode was selected. In this mode, the deprotonated molecular ions $[M–H]^-$ appeared at *m/z* 481. As adduct ions were not abundant, they were not investigated further. To select a product ion, product ion spectra of *m/z* 481 were obtained, and abundant product ions were further investigated in terms of sensitivity and response linearity. The transition of *m/z* 481→*m/z* 234 was chosen for monitoring, as this transition gave excellent response linearity. MS/MS parameters were optimized to obtain greater ion intensity.

Next, HPLC conditions under which conformers form a single HPLC peak were investigated in accordance with the total concentration workflow. In this investigation, ASP2151 neat solutions were analyzed using LC-MS/MS, not column-switching HPLC. A mobile phase was delivered at a flow rate of 0.8 mL/min to a YMC-Pack Pro C18 column (4.6 mm I.D. × 50 mm L, 3 μm) heated at 40°C, unless stated otherwise. As the ionization mode was APCI, a flow rate of 0.8 mL/min was considered reasonable, and an analytical column of 4.6 mm I.D. was selected. Column length of 50 mm and particle size of 3 μm were selected, as the author's general experiences in LC-MS/MS methods for small molecule drugs suggest that this column length and particle size are a good starting point. In the initial experiment, the author used acetonitrile/20 mmol/L ammonium acetate (40:60, v/v) as a mobile phase and obtained bimodal peaks derived from the two conformers of ASP2151 (Fig. 2-14(a)). The *k* were 3.9 for the *Z* form and 5.0 for the *E* form, and the α was 1.3. In accordance with the total concentration workflow, an elevated column temperature (60°C)

was tested with a mobile phase of acetonitrile/20 mmol/L ammonium acetate (35:65, v/v). The observed peak (Fig. 2-14(b)) was a single but fronting, ugly peak (S: 0.68 and *N*: 4099) with *k* of 6.6. As this was not satisfactory (S and *N* were expected to be around 1.0 and greater than 5600, respectively), the author decided to continue the investigation. In accordance with the workflow, the mobile phase was changed from a neutral mobile phase to an acidic one. In particular, acetonitrile/0.1% formic acid (40:60, v/v) was used. The obtained mass chromatogram (Fig. 2-14(c)) had bimodal peaks whose *k* were 3.8 and 5.0 (α of the two peaks was 1.3) and was nearly identical to those when using a neutral mobile phase. Organic modifiers in the mobile phase were then changed. Methanol was tested first, as THF added at a high concentration is detrimental to ionization and incompatible with ordinate HPLC components such as PEEK tubes. When ASP2151 was chromatographed with a mobile phase of methanol/20 mmol/L ammonium acetate (55:45, v/v) at a column temperature of 40°C, *k* were 4.8 and 5.3, and α was 1.1 (Fig. 2-14(d)), revealing that the two conformers eluted more closely using a methanol-based mobile phase than an acetonitrile-based mobile phase. Based on these data, methanol/THF/20 mmol/L ammonium acetate (45:5:50, v/v/v) at a column temperature of 40°C was tested. Under this condition, ASP2151 was eluted as a single peak whose S was 0.95 and *N* was 8838, indicating a symmetrical and sharp peak (Fig. 2-14(e)). *k* was 5.1, which provides a good balance between run time and peak separation. Therefore this condition was used for analytical HPLC.

Further, pretreatment was examined. In accordance with the total concentration workflow, a column-switching method was selected. To establish a column-switching method, several extraction columns were tested prior to final selection. The extraction columns were required to retain ASP2151 well during the separation of ASP2151 and endogenous large molecules and release ASP2151 rapidly during elution. Of the columns examined, the MAYI-ODS, which is packed with restricted-access materials (Hashi et al., 2004; Yang et al.,

2013), provided the best performance. In addition, the MAYI-ODS showed negligible lot-to-lot variation and excellent durability against the injection of hundreds of biological samples (data not shown). The author therefore selected the MAYI-ODS as an extraction column.

To obtain an adequate analyte response at the expected LLOQ and allow re-injection of the injection sample, the injection volume was set at 50 μL. The initial mobile phase for the extraction column was a mixture of 20 mmol/L ammonium acetate and acetonitrile (95:5, v/v). As the buffer was the same as the mobile phase for the analytical column, the approach to equilibrium of the extraction column was considered rapid. Due to the low acetonitrile concentration of the mobile phase, the analyte was retained at the entrance of the extraction column, and endogenous proteins passed through the extraction column without protein precipitation. After separation from endogenous large molecules, ASP2151 should elute from the extraction column. Based on the results, the author delivered the analytical mobile phase to the extraction column at 0.8 mL/min for 2.0 min. The extraction column was washed with acetonitrile/water (50:50, v/v). This prolonged the life of both the extraction column and analytical column.

Finally, the stability of ASP2151 in dog plasma and urine was assessed as a part of pre-validation, in line with the analyte stabilization workflow. ASP2151 was stable in dog plasma and urine at room temperature for 24 h and after three freeze-thaw cycles. This was verified in a validation study (see “Method validation for ASP2151”). Based on the analyte stabilization workflow, no additional measures for stabilization were examined.

Method validation for ASP2151

Typical chromatograms of the blank and spiked samples are shown in Fig. 2-15. No interfering peaks were observed at the retention times of the analyte or IS. ASP2151 in plasma

met the acceptance criteria with a CV of 3.2% and RE of 0.2% at the LLOQ of 5 ng/mL. ASP2151 in urine met the acceptance criteria with a CV of 2.0% and RE of 0.0% at the LLOQ of 0.5 μg/mL. This sensitivity enables the full evaluation of ASP2151 pharmacokinetic profiles, as the plasma and urine concentrations of ASP2151 were relatively high in planned nonclinical studies.

Calibration curves were linear over the concentration range of 5 to 1000 ng/mL in dog plasma and 0.5 to 100 μg/mL in dog urine with a coefficient of determination (R^2) of >0.998. A typical equation of a calibration curve for plasma is as follows:

$$y = 0.01x + 0.0008$$

where y denotes the peak area ratio of ASP2151 to IS and x denotes the plasma concentration. Analyte peak shapes were the same at all concentrations tested.

Results of accuracy and precision for the plasma method are summarized in Table 2-2. Similar results were obtained for the urine method. In plasma, the inter-day accuracies in RE ranged from −4.4% to 1.2% and the inter-day precision in CV was ≤4.3%. In urine, the inter-day accuracies in RE ranged from −3.9% to 4.7% and the inter-day precision in CV was ≤7.8%. These results met the acceptance criteria. In addition, the accuracy and precision were consistent at analyte concentrations tested in both matrices, suggesting that concentration data can be delivered in a reproducible and reliable manner independent of analyte concentration.

Results of stability for the plasma method are shown in Table 2-3. Similar results were obtained for the urine method. Changes after storage or handling were within −9.0% and 12.0% and considered acceptable. ASP2151 was stable in dog plasma and urine at room temperature for 24 h and after three freeze-thaw cycles. ASP2151 was stable at −20°C for 183 days in plasma and 82 days in urine. In addition, the post-preparative stability of ASP2151 was 48 h in both matrices. As no stability issue was noted, stabilization was not necessary with ASP2151.

These validation results suggest that the total concentration workflow worked well with ASP2151.

Application to pharmacokinetic study of ASP2151

The validated methods were used to determine the plasma and urine concentration of ASP2151 in dogs. Fig. 2-16 shows the mean plasma concentration-time profiles of ASP2151 following a single oral administration. The plasma concentration was quantifiable for up to 24 h after administration, and pharmacokinetic parameters, including urinary excretion data, are shown in Table 2-4 and Table 2-5. No problems were noted with the analysis, including conversion of non-analyte to ASP2151 in the study samples. These results suggest that the validated methods are applicable to pharmacokinetic studies in dogs and that the absence of a problematic non-analyte was well predicted using the interference management workflow. The precise prediction saved time and resources for an animal experiment, without running the risk that a non-analyte would convert to the analyte during sample collection, storage, and analysis of study samples. Pharmacokinetic analysis revealed the following properties of ASP2151 in dogs: (1) after a single i.v. administration at 1 mg/kg, the plasma concentration of ASP2151 decreased in a monophasic manner with an elimination half-life ($t_{1/2}$) of 9.69 h; (2) urinary excretion as unchanged drug was 8%; (3) absolute bioavailability (BA) ranged from 21.85% to 33.13% at doses of 0.3, 1, and 3 mg/kg; (4) after a single oral administration, maximum plasma concentration (C_{max}) was observed at 2.50 to 3.50 h, indicating rapid absorption; and (5) ASP2151 plasma concentration increased with oral dosage. The rapid absorption and dose-dependent increase in exposure were considered favorable absorption properties for a drug candidate. Urinary excretion was determined to be a relatively minor elimination pathway in dogs.

2.3.3 Application to ASP3258

Method development for ASP3258

An overview of method development for ASP3258 is shown in Fig. 2-17. First, it was determined whether the ASP3258 method is within the scope of the total concentration workflow. As ASP3258 does not have conformers, it was judged that the ASP3258 method is out of the scope of the total concentration workflow.

In accordance with the interference management workflow, the author examined whether an interfering compound is likely to be present in *in vivo* samples, and if so, into what compound class it is classified. ASP3258 was classified into category 1 (carboxylic acid) and category 2 (tertiary amine). The workflow suggested examining the presence of ASP3258-AG in *in vivo* samples. Therefore, ASP3258 was orally administered to rats and plasma samples were collected and analyzed. Neither the LLE nor SPE method detected ASP3258-AG, clearly indicating that the conversion of ASP3258-AG to ASP3258 cannot occur in rat plasma. In addition, the workflow specified reference to *in vitro* metabolism data to check the presence of the *N*-oxide. However, the *N*-oxide was not found in available *in vitro* drug metabolism data, and the author accordingly decided not to examine the presence of *N*-oxide *in vivo*.

As described in “General Introduction”, fluorescence detection was selected for the ASP3258 method. To reproducibly obtain a symmetrical peak for ASP3258, the author examined columns, mobile phases, and reconstitution solutions. As ASP3258 is an acid, column screening was carried out using an acidic mobile phase (i.e. a mixture of acetonitrile and acetic acid aqueous solution). Of the columns examined, the Shiseido Capcell Pak C18 UG120 was the most promising. With an optimized mobile phase composition of acetonitrile/0.5% acetic acid (50:50, v/v), ASP3258 reproducibly showed a symmetrical peak (S: 1.17). The k was 4.9, which generally indicates a good balance between run time and peak

separation. Reconstitution solutions and injection volumes were examined to attain assay sensitivity without compromising the shape of the peak. As a result, the reconstitution solution consisted of acetonitrile/0.5% acetic acid (30:70, v/v), and the injection volume was 0.05 to 0.08 mL. The final HPLC method provided symmetrical peaks and sensitivity with complete separation of ASP3258 and IS.

A variety of SPE sorbents (C18, C8, C6, C4, C2, cyclohexyl, phenyl, or cyanopropyl-bonded silica sorbent and OASIS HLB sorbent) were tested. The optimized extraction procedure using C18-bonded silica sorbent exhibited a reproducible, high recovery rate, and selectivity.

The stability of ASP3258 in rat plasma was assessed as a part of pre-validation, in line with the analyte stabilization workflow. ASP3258 was stable in rat plasma at room temperature for 24 h and after three freeze-thaw cycles. This was verified in a validation study (see section “Method validation for ASP3258”). Based on the analyte stabilization workflow, no additional measures for stabilization were examined.

Method validation for ASP3258

Typical chromatograms of blank and spiked plasma samples are shown in Fig. 2-18(a) and (b) and demonstrate the selectivity of the method. Given that precision at 2.5 ng/mL was 2.64% and accuracy was 3.22%, the LLOQ for ASP3258 in plasma was 2.5 ng/mL, and met the acceptance criteria. Calibration curves were linear over the concentration range of 2.5 to 250 ng/mL in rat plasma with R^2 of >0.998. A typical equation of the calibration curve is as follows: y=0.01339x−0.00082, where y represents the peak height ratio of ASP3258 to IS and x the plasma concentration. The inter-day accuracies in RE ranged from 1.57% to 5.24%. The inter-day precision in CV was ≤3.50%. These results met the acceptance criteria. Extraction recovery of ASP3258 was 92.68% and 95.27% at

7.5 ng/mL and 200 ng/mL, respectively, indicating that extraction recovery was constant at a wide range of concentrations. Extraction recovery of IS was 90.19%, which is similar to ASP3258. Changes in ASP3258 concentration after storage or handling were within −0.50% and 10.63% and considered acceptable. ASP3258 was stable in rat plasma at room temperature for 24 h, at −20°C for 60 days, and after three freeze-thaw cycles. In addition, the post-preparative stability of ASP3258 was 48 h at 4°C. As no stability issue was noted, stabilization was not necessary with ASP3258.

Application to pharmacokinetic study of ASP3258

A typical chromatogram of a pharmacokinetic study sample is shown in Fig. 2-18(c). The method was sensitive enough to quantify concentrations up to 24 h after administration (Fig. 2-19) and enabled calculation of the pharmacokinetic parameters using mean plasma concentrations (Table 2-6). These results suggest that the validated method is applicable to pharmacokinetic studies in rats. No problems were noted with the analysis, including conversion of non-analytes to ASP3258 in the study samples. The absence of AG and *N*-oxide in rat plasma was consistent with the observation in an extensive drug metabolism study in rats which was conducted in parallel with this pharmacokinetic study (Ohtsu et al., 2015). These results suggest that the validated methods are applicable to pharmacokinetic studies in rats and that the interference management workflow worked well with ASP3258.

Pharmacokinetic analysis revealed the following properties of ASP3258 in rats: 1) $t_{1/2}$ of ASP3258 was 5.78 h, which is a relatively long $t_{1/2}$ in rats and suggests that the efficacy of ASP3258 may be sufficiently long; and 2) volume of distribution at steady state (Vd_{ss}) was 1185 mL/kg, suggesting extensive tissue distribution. As ASP3258 exerts its efficacy in respiratory organs, not in plasma or blood, the extensive tissue distribution is also an encouraging result, from viewpoint of drug development.

2.3.4 Application to enzalutamide

Method development for enzalutamide and its metabolites

An overview of method development for enzalutamide, M1, and M2 is shown in Fig. 2-20. The method for the three analytes was first confirmed to be within the scope of the total concentration workflow. As none of the analytes has conformers, it was judged that the method is out of the scope of the total concentration workflow.

In accordance with the interference management workflow, the author then examined whether an interfering compound is likely to be present in *in vivo* samples, and if so, what compound class it is classified into. M1 was classified into category 1 (carboxylic acid) and category 4 (metabolite of a convertible drug [enzalutamide] and metabolite of a convertible precursor metabolite [M2], respectively). The workflow suggested examining the presence of M1 AG in *in vivo* samples. The available drug metabolism data clarified the absence of AG of M1 in plasma of rats, dogs, and humans (Bennett et al. 2014; Ohtsu et al. in press), suggesting the absence of AG of M1 in mouse plasma. The workflow also suggested examining the conversion of enzalutamide and M2 to M1 during stability tests in pre-validation, as hydrolysis of enzalutamide and M2 at the amide bonds would produce M1. The author confirmed that the three analytes had no additional issues. In conclusion, based on the interference management workflow, it was highly recommended to investigate the degradation of enzalutamide and M2 into M1.

When MS/MS conditions were optimized per analyte, quantification of three different components (enzalutamide, M1, and M2) in the same wide calibration ranges (2500-fold) and in the same run was not possible. This was overcome by further modification of MS/MS conditions. Given that the monitoring ion for M1 was close to that of M2, M2 appeared as an interfering peak in M1 mass chromatograms. This selectivity issue was solved

by chromatographic separation of M1 and M2. Significant carry-over was observed for all analytes. Changes in autosampler washing solvent or autosampler device did not improve results, suggesting that the observed carry-over was caused by other sources. The author suspected column carry-over (Hughes et al., 2007), which is caused by analyte residues on HPLC columns. Williams et al. (Williams et al., 2012) and Vu et al. (Vu et al., 2013) overcame carry-over in their studies by changing HPLC columns. Several HPLC columns were tested and carry-over was found to be negligible using the Cadenza CD-C18 column.

Bennett et al. reported a method for human plasma using a basic buffer that had an extraction recovery of 15.5% to 22.1% for M1 (Bennett et al., 2014). As M1 is a carboxylic acid, the author improved recovery by using 0.1% formic acid in water.

As stability tests of pre-validation, degradation of enzalutamide and M2 into M1 was examined following the suggestion from the interference management workflow. While enzalutamide was found to be stable in intact mouse plasma, a decrease in M2 concentration and increase in M1 concentration was noted when stored in intact mouse plasma at room temperature (Table 2-7). In accordance with the analyte stabilization workflow, the author examined the chemical structure, photosensitivity, interconvertible stereoisomers, and MOA and speculated that the conversion of M2 to M1 can be ascribed to hydrolysis of the amide moiety of M2. The matrix in question is plasma, which has a neutral pH and abundant esterase content. Taken together, it was estimated that the cause of M2 degradation might be esterase-mediated metabolism of M2 to M1. In accordance with the analyte stabilization workflow, cooling was used to stabilize M2, but this was not sufficient (Table 2-7). The esterase inhibitor DDVP was therefore added to plasma, which afforded acceptable bench-top stability (see “Method validation for enzalutamide and its metabolites”). As no conversion of enzalutamide and M2 to M1 can occur under the established storage condition, no further action was suggested by the interference management workflow.

Method validation for enzalutamide and its metabolites

No interfering peaks in mouse plasma were observed at the retention times of the analytes or IS. Peak area ratios of the analytes to IS were linear against analyte concentrations over a concentration range of 20 to 50000 ng/mL in mouse plasma. Calibration curves were constructed with R^2 ≥0.994. The inter-day accuracies in RE ranged from −8.0% to 4.6%. The inter-day precision in CV was ≤9.8%. These results met the acceptance criteria. Results of stability are shown in Table 2-8. Changes after storage or handling were within −8.8% and 10.7% and were considered acceptable. Enzalutamide and its two metabolites were stable in DDVP-added mouse plasma at 4°C for 24 h, at −20°C for approximately six months, and after 3 freeze-thaw cycles. The post-preparative stability of analytes was 50 h. Partial validation due to changes in IS was conducted and all results met the acceptance criteria.

Application to pharmacokinetic studies of enzalutamide

The validated methods were used to determine the plasma concentrations of enzalutamide, M1, and M2 in mice. The plasma concentration profile in mice is shown in Fig. 2-21. Pharmacokinetic parameters are shown in Table 2-9. No problems were noted with the analysis, including conversion of non-analytes to enzalutamide, M1, and M2 in the study samples. These results suggest that the validated method is applicable to pharmacokinetic studies in mice and that the interference management workflow worked well with enzalutamide, M1, and M2. In this case, the combination of the interference management workflow and the extensive drug metabolism data identified the problematic non-analytes (e.g. enzalutamide and M2 in quantification of M1) efficiently, and saved time and resource requirements in an animal experiment, without the risk that non-analytes would convert to the analytes during sample collection, storage and analysis of study samples.

After repeated oral administration of enzalutamide to mice, the C_{max} of enzalutamide occurred at 4 to 8 h postdose. The C_{max} and area under the plasma concentration-time curve (AUC) of enzalutamide increased with increasing dose within the dose range examined but the increases were less than dose-proportional above 30 mg/kg/day. Sex differences in exposure were not apparent. These exposure data will be used during assessment of time-dependency, dose-dependency, and sex differences in safety in mice. M1 and M2 plasma concentrations on Day 28 ranged from 6.17% to 21.20% of enzalutamide, suggesting that enzalutamide is likely the most important component among the three components in *in vivo* testing in mice. Plasma concentrations of enzalutamide, M1, and M2 tended to be higher after repeated administration than on Day 1, indicating similarity between mice and humans regarding the effect of multiple dosing on pharmacokinetics, given that an increase in plasma concentration after repeated administration was also observed in humans (Gibbons et al., 2015b).

2.4 Conclusion from chapter 2

The author developed three workflows. The first workflow was to develop a determination method for the total concentration of conformers. The second workflow was to stabilize a convertible analyte. The third workflow was to manage interference from a non-analyte that may convert to an analyte.

After the development of the workflows, bioanalytical methods for ASP2151, ASP3258, enzalutamide and enzalutamides' metabolites (M1 and M2) were developed using the workflows, validated in accordance with FDA guidance (FDA, 2001), and applied to pharmacokinetic studies. The workflows were evaluated using the performance of the method in method validation and sample analysis and proved to be effective. In addition, the measured concentration data were used for pharmacokinetic assessment of these analytes.

Table 2-1. Time program and flow rates for a column-switching HPLC system for ASP2151.

Time (min)	Flow rate (mL/min)			Valve position
	Pump A	Pump B	Pump C	
	Mobile phase A	Mobile phase B	Mobile phase C	
0.00	2.0	0.0	0.8	A
2.50	2.0	0.0	0.8	A→B
2.51	0.0	2.0	0.8	B
4.50	0.0	2.0	0.8	B→A
7.50	0.0	2.0	0.8	A
7.51	2.0	0.0	0.8	A
10.00	2.0	0.0	0.8	A

Mobile phase A: acetonitrile/20 mmol/L ammonium acetate (5:95, v/v)

Mobile phase B: acetonitrile/water (50:50,v/v)

Mobile phase C: methanol/THF/20 mmol/L ammonium acetate (45:5:50, v/v/v)

Table 2-2. Accuracy and precision of a bioanalytical method for ASP2151 in dog plasma.

QC sample	Nominal concentration (ng/mL)	Intra-day (n=6)		Inter-day (n=18 [n=6 on 3 days])	
		RE (%)	CV (%)	RE (%)	CV (%)
Low	15	−1.6	5.4	1.2	4.3
Medium	75	−1.3	2.8	−0.7	2.1
High	750	−5.4	0.9	−4.4	1.5

CV, coefficient of variation; QC, quality control; RE, relative error

Note: Accuracy was expressed as RE and precision as CV.

Table 2-3. Stability of ASP2151 in dog plasma or injection samples.

Storage or handling	QC sample	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Stability (%)
Short-term[a)]	Low	15	15.69	4.6
	High	750	723.74	−3.5
Long-term[b)]	Low	15	14.74	−1.7
	High	750	700.64	−6.6
Freeze-thaw[c)]	Low	15	16.11	7.4
	High	750	766.26	2.2
Post-preparative[d)]	Low	15	15.95	6.3
	High	750	751.32	0.2

QC, quality control

a) 24 h at room temperature, b) 183 days at −20°C, c) three cycles, d) 48 h at 4°C

Table 2-4. Pharmacokinetic parameters of ASP2151 after a single i.v. administration to dogs (1 mg/kg).

Parameter	Value (mean ± standard deviation)
CL_{tot} (L/h/kg)	0.13 ± 0.02
Vd_{ss} (L/kg)	1.74 ± 0.05
AUC_t (μg × h/mL)	6.43 ± 0.78
AUC_{inf} (μg × h/mL)	7.87 ± 1.35
$t_{1/2}$ (h)	9.69 ± 1.54
f_e	0.08 ± 0.03
CL_R (L/h/kg)	0.01 ± 0.00

AUC_t, area under the plasma concentration-time curve from time 0 to last quantifiable time point; AUC_{inf}, area under the plasma concentration-time curve from time zero to infinity; CL_R, renal clearance; CL_{tot}, total body clearance; f_e, ratio of renal excretion as unchanged drug; $t_{1/2}$, elimination half-life; Vd_{ss}, volume of distribution at steady state

Table 2-5. Pharmacokinetic parameters of ASP2151 after a single oral administration to dogs (0.3, 1, and 3 mg/kg).

Dose (mg/kg)	0.3	1	3
Parameter	**Value (mean ± standard deviation)**		
C_{max} (ng/mL)	31.29 ± 9.93	98.27 ± 12.05	450.54 ± 182.78
T_{max} (h)	2.50 ± 1.00	3.50 ± 1.00	3.50 ± 1.00
AUC_t (μg × h/mL)	0.41 ± 0.18	1.45 ± 0.21	6.54 ± 2.56
AUC_{inf} (μg × h/mL)	0.51 ± 0.16	1.76 ± 0.29	7.87 ± 3.28
$t_{1/2}$ (h)	8.78 ± 1.39	9.18 ± 1.02	8.61 ± 1.13
BA (%)	21.85 ± 7.32	23.08 ± 6.73	33.13 ± 10.97

AUC_t, area under the plasma concentration-time curve from time 0 to last quantifiable time point; AUC_{inf}, area under the plasma concentration-time curve from time zero to infinity; BA, bioavailability; C_{max}, maximum plasma concentration; T_{max}, time to reach maximum plasma concentration; $t_{1/2}$, elimination half-life

Table 2-6. Pharmacokinetic parameters of ASP3258 after a single i.v. administration to rats (0.1 mg/kg).

Parameter	Value
CL_{tot} (mL/h/kg)	169
Vd_{ss} (mL/kg)	1,185
AUC_{24} (ng × h/mL)	563
AUC_{inf} (ng × h/mL)	591
$t_{1/2}$ (h)	5.78

AUC_{24}, area under the plasma concentration-time curve from time 0 to 24 h; AUC_{inf}, area under the plasma concentration-time curve from time zero to infinity; CL_{tot}, total body clearance; $t_{1/2}$, elimination half-life; Vd_{ss}, volume of distribution at steady state

Table 2-7. Effect of temperature on stability of enzalutamide, M1, and M2 in intact mouse plasma.

Analyte	Temperature	Nominal concentration (ng/mL)	Measured concentration (ng/mL)[a)]	Stability (%)
Enzalutamide	Room temperature	60	59.14	-1.4
		40,000	41,929.75	4.8
	4°C	60	59.51	-0.8
		40,000	41,968.68	4.9
M1	Room temperature	60	85.71	42.9
		40,000	53,643.07	34.1
	4°C	60	69.92	16.5
		40,000	41,637.05	4.1
M2	Room temperature	60	37.77	-37.0
		40,000	29,626.31	-25.9
	4°C	60	50.73	-15.4
		40,000	38,838.64	-2.9

a) Mean (n=3)

Duration: 2 h

Table 2-8. Stability of enzalutamide, M1, and M2 in DDVP-added mouse plasma or extracts.

Analyte	Storage or handling	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Stability (%)
Enzalutamide	Short-term[a)]	60	58.34	-2.8
		40,000	36,646.23	-8.4
	Long-term[b)]	60	66.41	10.7
		40,000	38,324.62	-4.2
	Freeze-thaw[c)]	60	61.52	2.5
		40,000	37,262.75	-6.8
	Post-preparative[d)]	60	61.39	2.3
		40,000	36,470.52	-8.8
M1	Short-term[a)]	60	61.68	2.8
		40,000	38,636.32	-3.4
	Long-term[b)]	60	65.50	9.2
		40,000	37,286.38	-6.8
	Freeze-thaw[c)]	60	56.58	-5.7
		40,000	38,180.09	-4.5
	Post-preparative[d)]	60	57.86	-3.6
		40,000	37,018.44	-7.5
M2	Short-term[a)]	60	66.14	10.2
		40,000	39,973.63	-0.1
	Long-term[b)]	60	61.01	1.7
		40,000	41,307.22	3.3
	Freeze-thaw[c)]	60	61.21	2.0
		40,000	36,601.80	-8.5
	Post-preparative[d)]	60	59.67	-0.5
		40,000	39,892.91	-0.3

a) 24 h at 4°C, b) 188 days at −20°C, c) 3 cycles, d) 50 h at 5°C

$^{13}CD_3$-labeled analogues were used as IS.

Table 2-9. Pharmacokinetic parameters of enzalutamide, M1, and M2 after 4-week repeated oral administration to mice.

Analyte	Dose level (mg/kg/day)	Sex	C_{max} (μg/mL)	T_{max} (h)	AUC_{24} (μg·h/mL)	AUC_{72} (μg·h/mL)	Metabolite ratio (%) [a)]	Accumulation index [b)]
Enza	10	Male	7.49	8	134.01	182.42	NA	1.54
		Female	8.34	8	124.97	146.66	NA	1.48
	30	Male	19.27	4	291.75	356.70	NA	1.12
		Female	22.39	8	356.20	411.28	NA	1.27
	60	Male	22.37	8	371.08	426.25	NA	0.75
		Female	32.73	8	512.17	587.23	NA	1.14
M1	10	Male	0.60	8	12.02	17.95	8.97	3.80
		Female	0.67	8	11.47	15.51	9.18	2.47
	30	Male	2.18	8	35.94	47.40	12.32	2.61
		Female	2.60	8	44.05	60.82	12.37	2.39
	60	Male	3.14	8	57.50	78.05	15.50	1.40
		Female	6.44	8	108.59	138.79	21.20	3.84
M2	10	Male	0.43	8	8.58	12.22	6.40	2.96
		Female	0.46	8	7.70	9.73	6.17	1.75
	30	Male	1.88	8	33.85	43.38	11.60	2.32
		Female	2.05	8	33.01	41.62	9.27	1.50
	60	Male	3.35	8	56.57	72.43	15.25	1.45
		Female	4.26	8	75.90	92.90	14.82	1.90

AUC_{24}, area under the plasma concentration-time curve from time 0 to 24 h postdose; AUC_{72}, area under the plasma concentration-time curve from time 0 to 72 h postdose; C_{max}, maximum plasma concentration; Enza, enzalutamide; NA, not applicable; T_{max}, time to reach maximum plasma concentration

a) (AUC_{24} of a metabolite)/(AUC_{24} of enzalutamide)×100; b) (Day 28 AUC_{24})/(Day 1 AUC_{24})

$[CD_3]_2$-labeled analogues were used as IS.

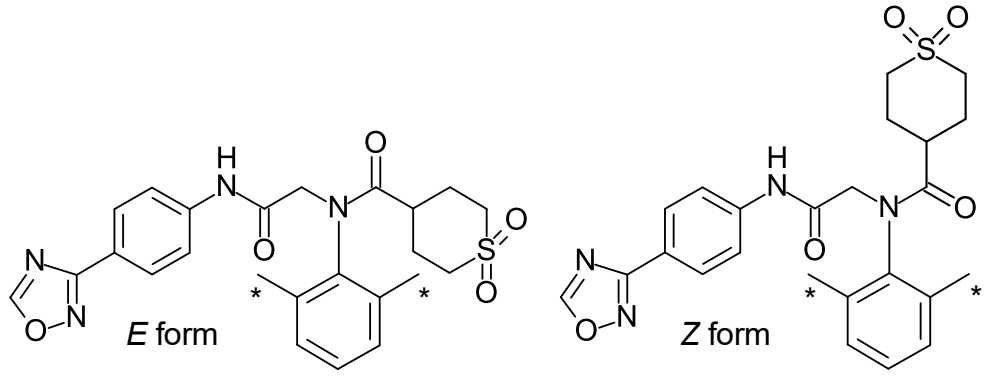

Fig. 2-1. Chemical structures of ASP2151. Asterisks indicate the positions of CD_3 in AS1709867-00.

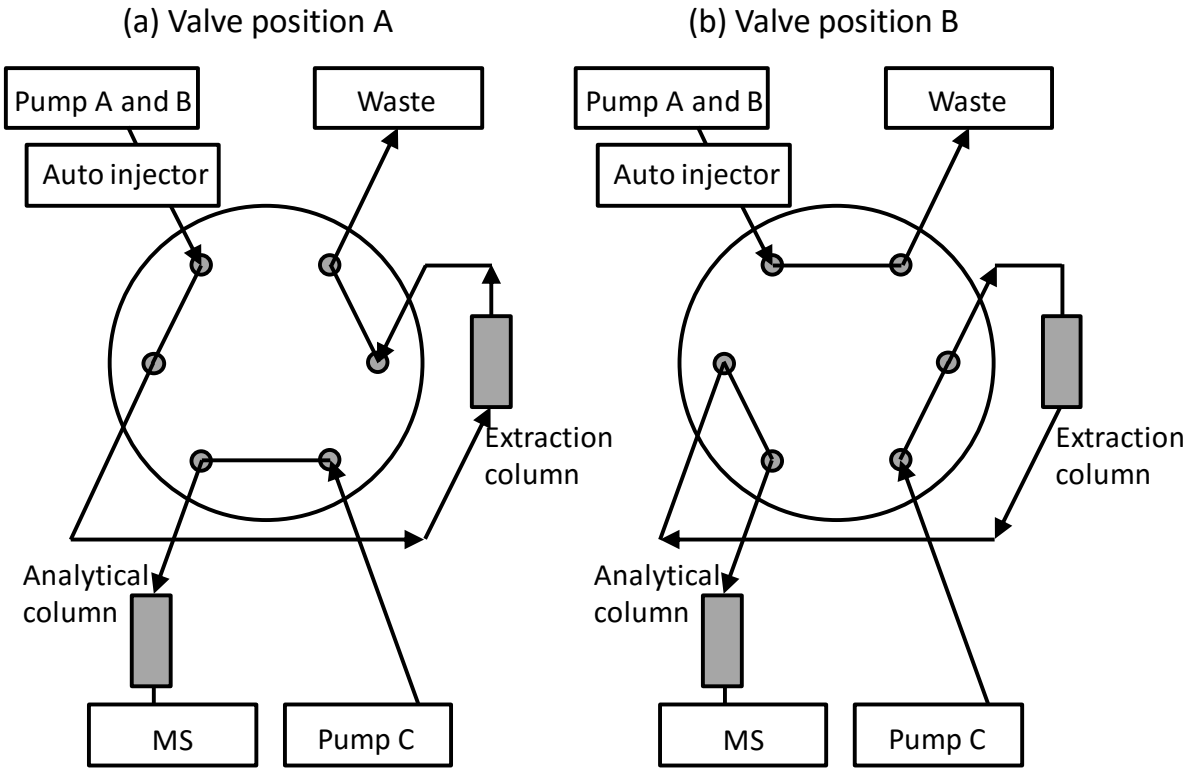

Fig. 2-2. Schematic diagram of the column-switching HPLC system for ASP2151. (a) The extraction column receives mobile phases from pump A and B. (b) The extraction column receives a mobile phase from pump C.

(a) ASP3258 (b) AS1406604-00

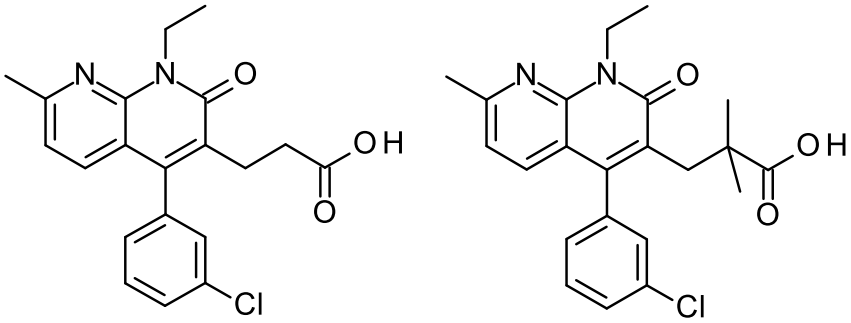

Fig. 2-3. Chemical structures of (a) ASP3258 and (b) AS1406604-00 (IS).

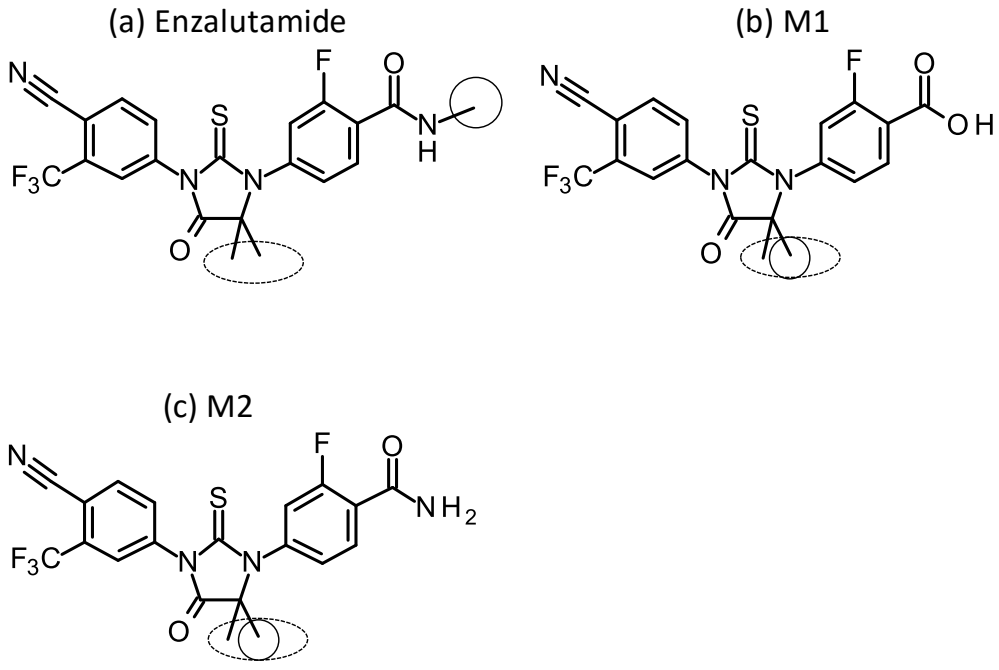

Fig. 2-4. Chemical structures of (a) enzalutamide, (b) its metabolite M1, and (c) M2. Circles indicate the position of $^{13}CD_3$ in the $^{13}CD_3$-labeled analogues. Dotted circles indicate the position of $[CD_3]_2$ in $[CD_3]_2$-labeled analogues.

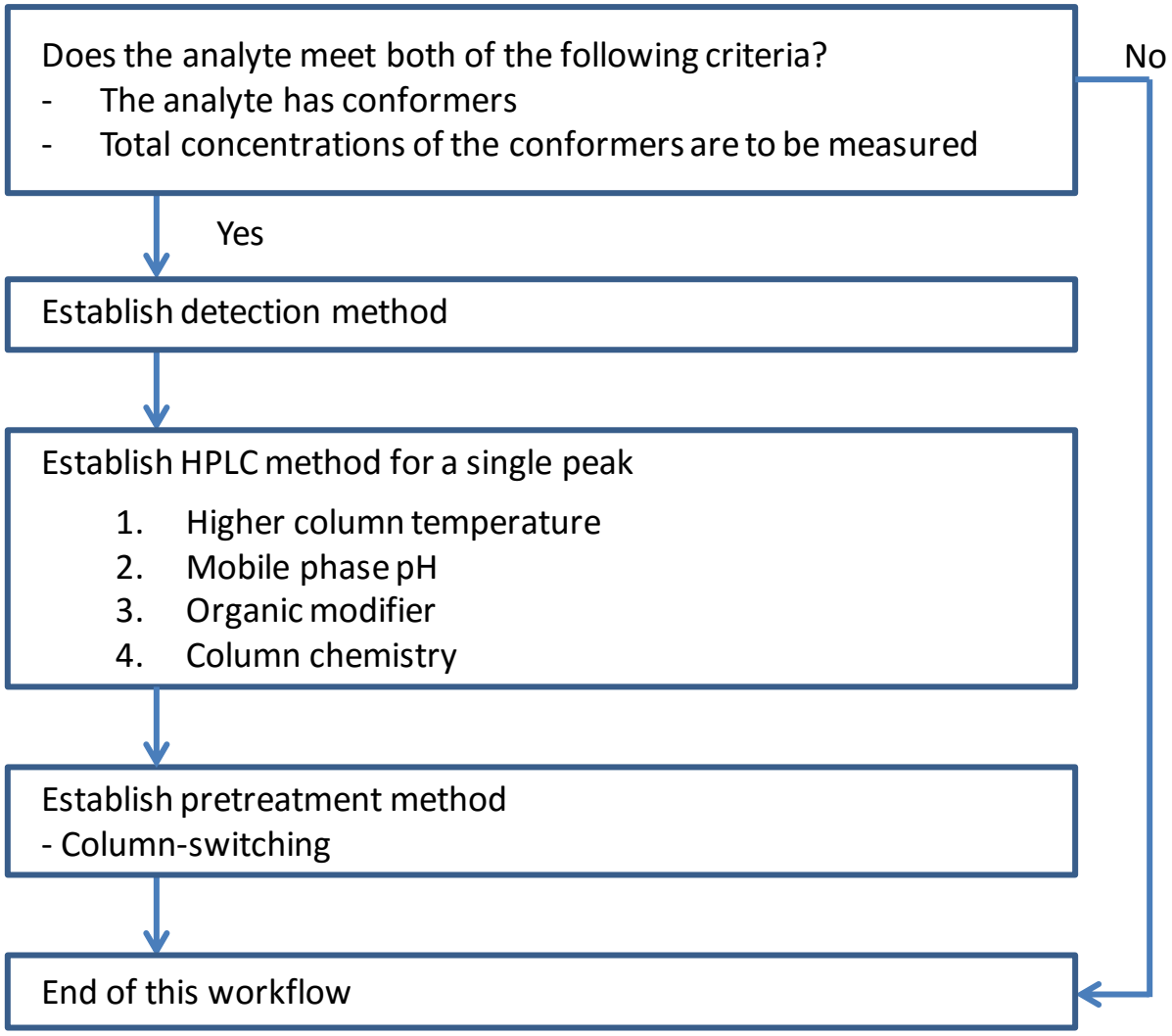

Fig. 2-5. Total concentration workflow.

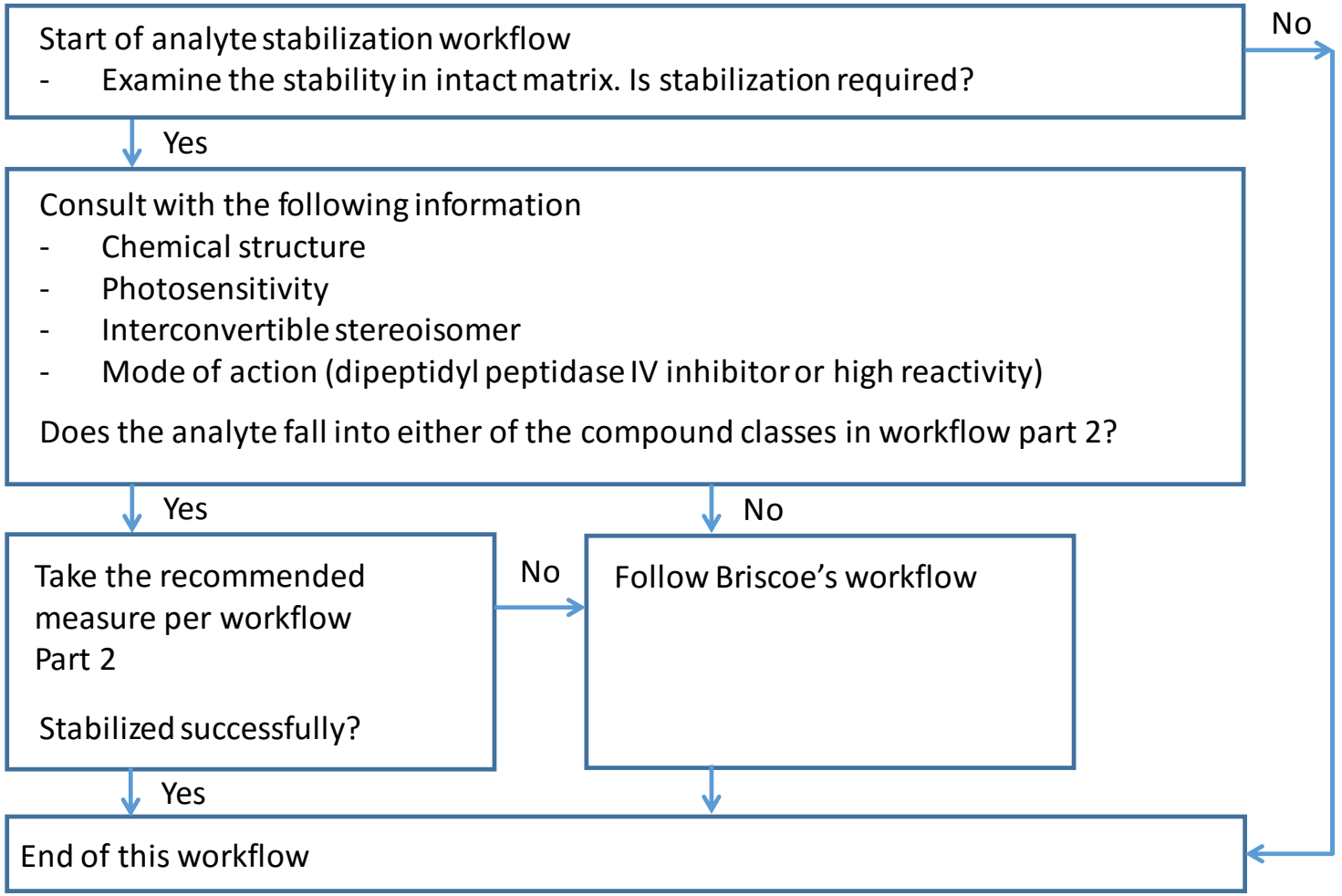

Fig. 2-6. Analyte stabilization workflow part 1: whole picture.

Identify the compound class for the analyte and take the recommended measure

Compound class	Recommended measure
Ester and amide	Reduced temperature, esterase inhibitor, pH (acidic) [a)]
Cytidine derivative	Cytidine deaminase inhibitor
Thiol	Derivatization
N-glucuronide	pH (acidic to neutral)
N-oxide	pH (acidic to neutral), protection from light
Phenol and alcohol	Protection from light, antioxidant, reduced temperature
Interconvertible stereoisomer	Reduced temperature, pH
Lactone and its hydroxycarboxylic acid	pH (weak acidic to neutral), reduced temperature
Photosensitive compound	Protection from light
Reactive compound	Derivatization, flash freezing
Dipeptidyl peptidase IV (DPP-IV) inhibitor	Another DPP-IV inhibitor

Not applicable or not stabilized

Stabilized successfully; end of workflow

Use Briscoe's workflow

Fig. 2-7. Analyte stabilization workflow part 2: typical compound classes. a) If the ester is an acyl glucuronide, pH (acidic) is more effective than esterase inhibitor.

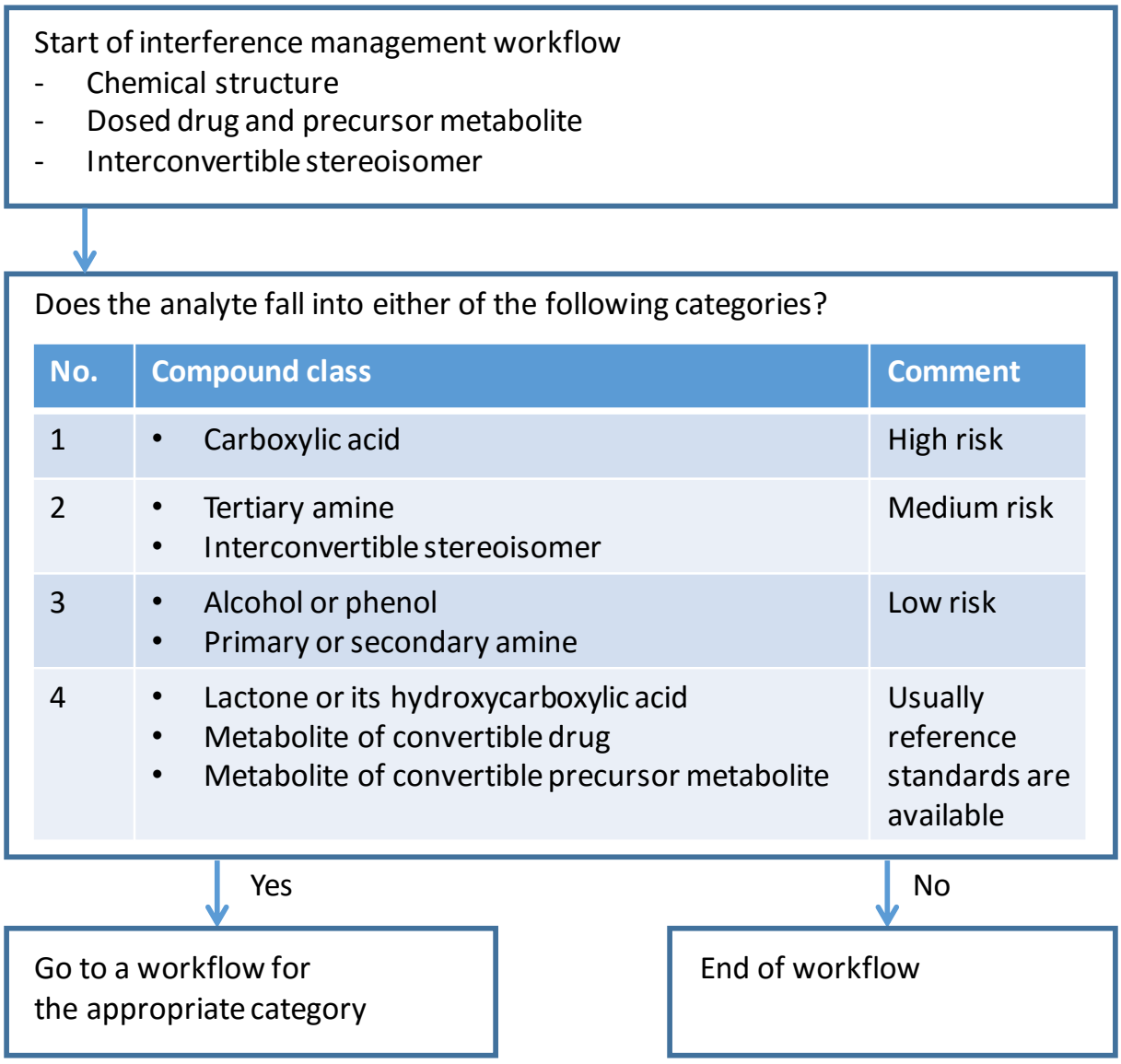

Fig. 2-8. Interference management workflow: categorization.

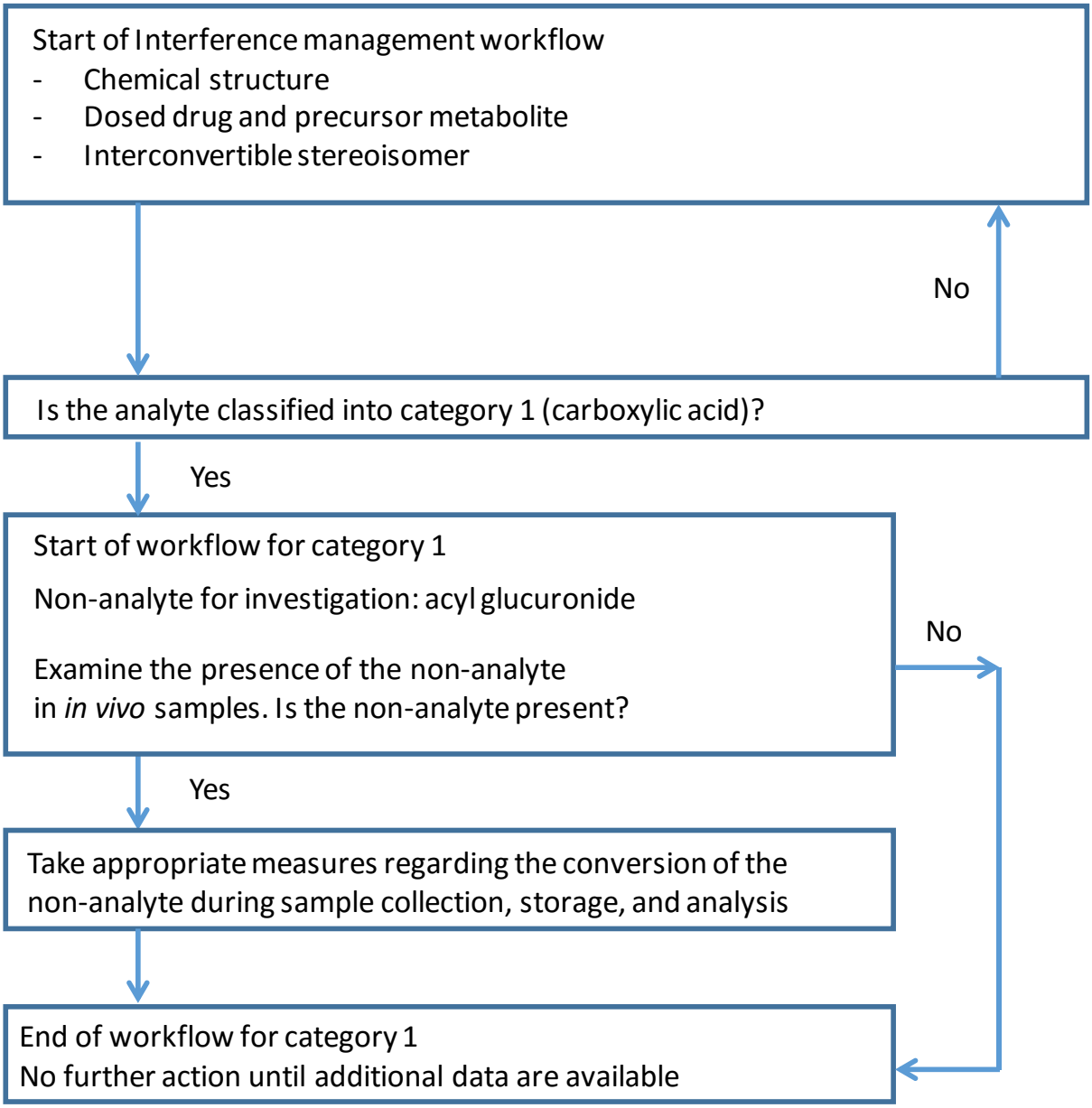

Fig. 2-9. Interference management workflow: Category 1.

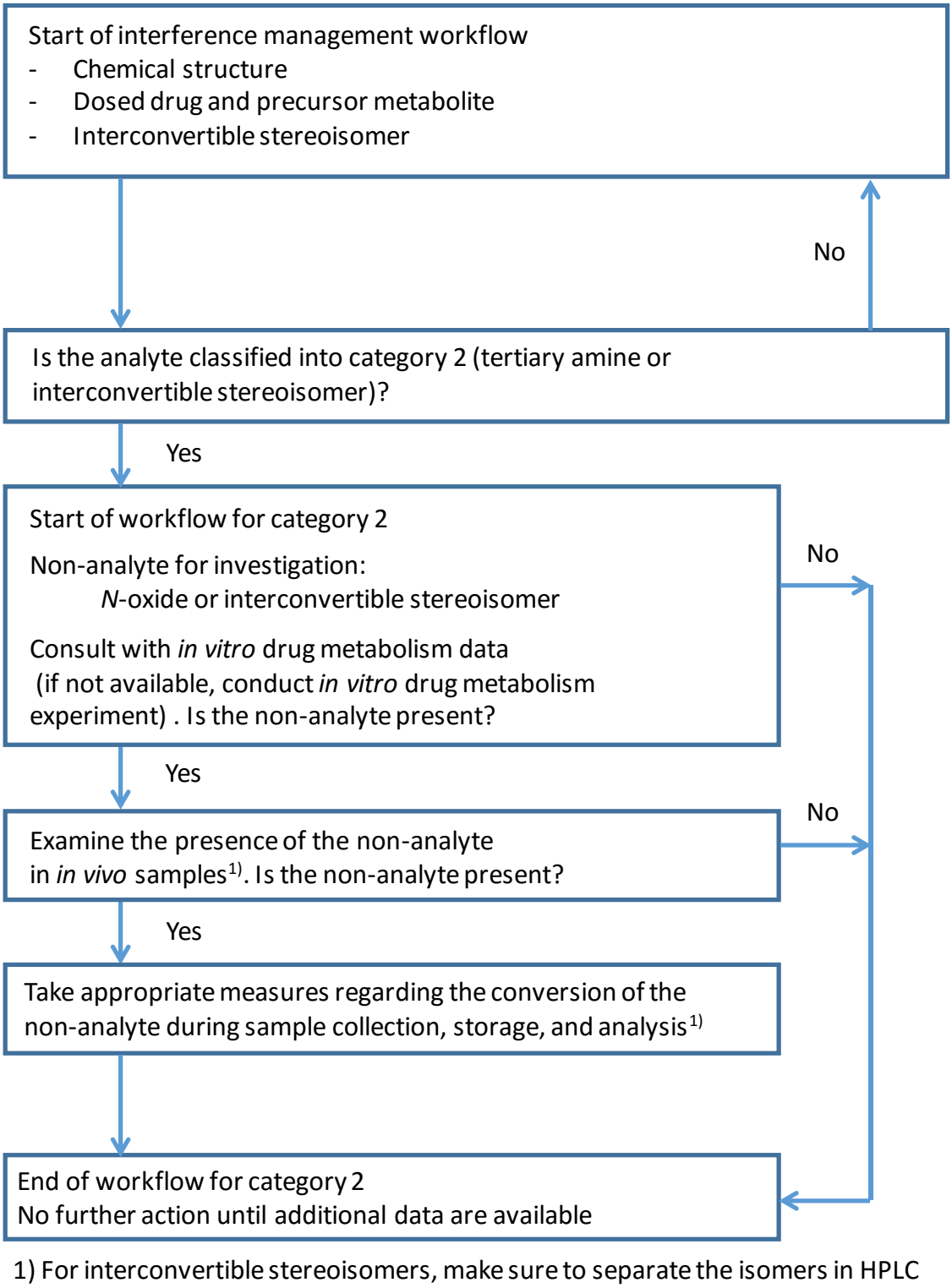

Fig. 2-10. Interference management workflow: Category 2.

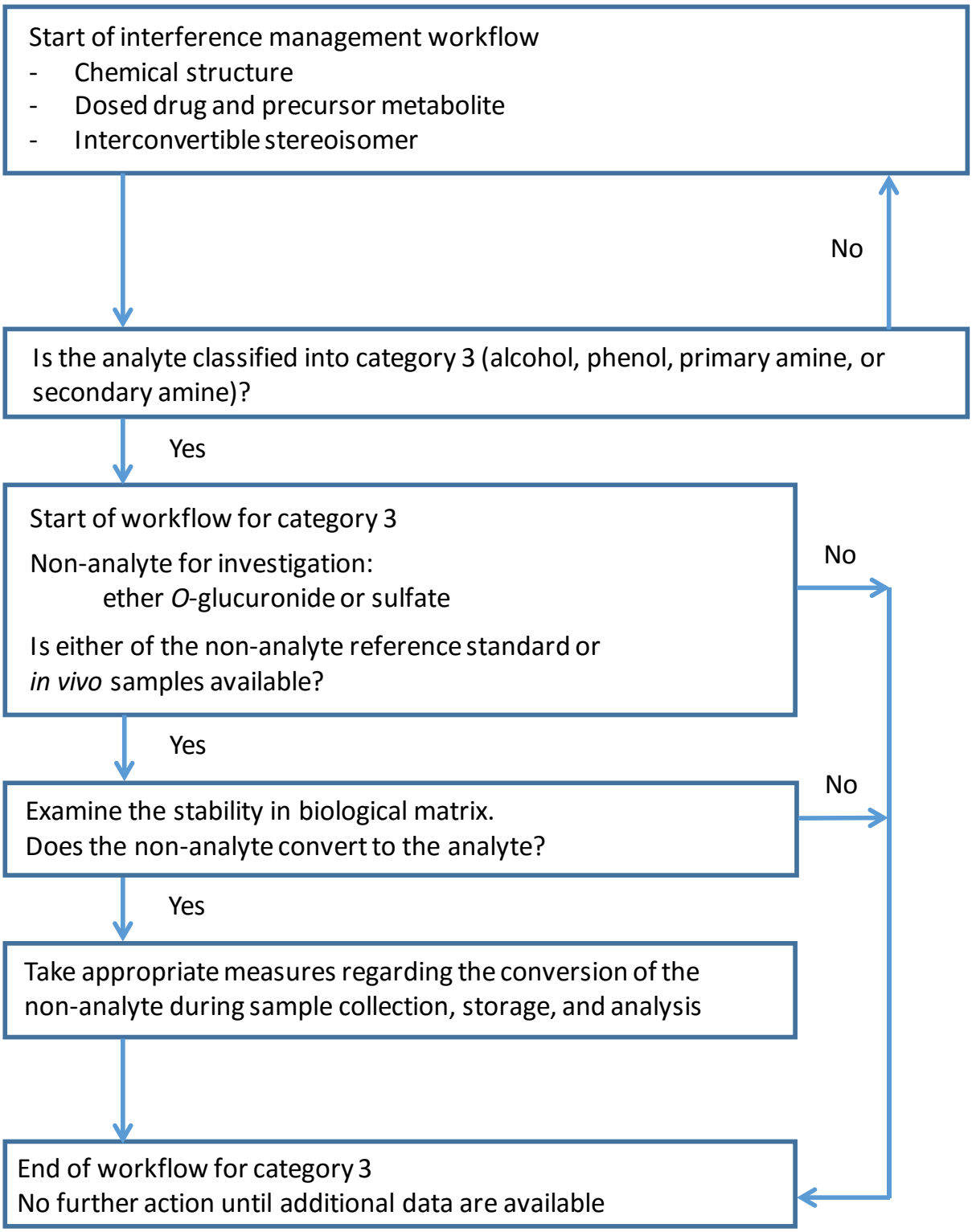

Fig. 2-11. Interference management workflow: Category 3.

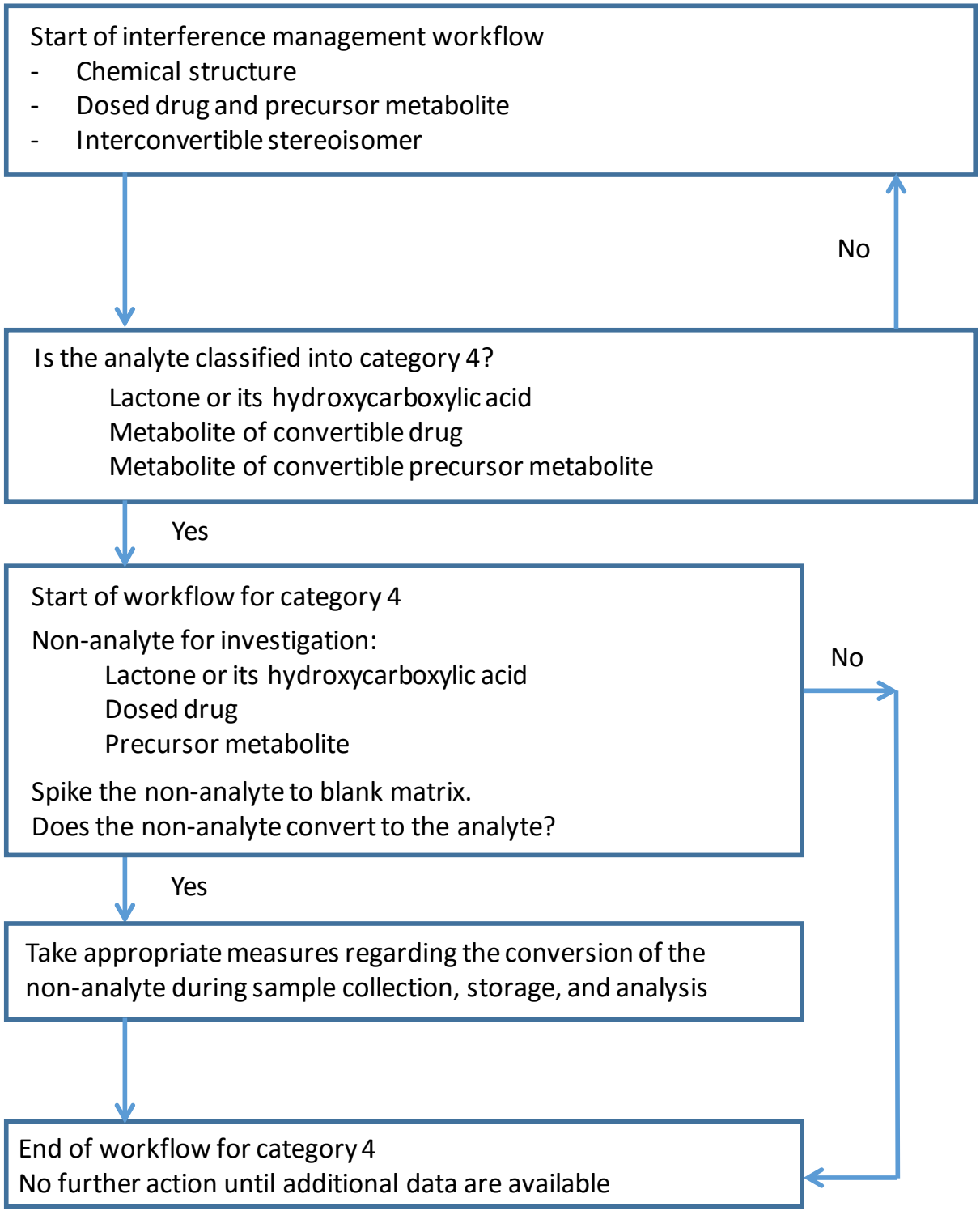

Fig. 2-12. Interference management workflow: Category 4.

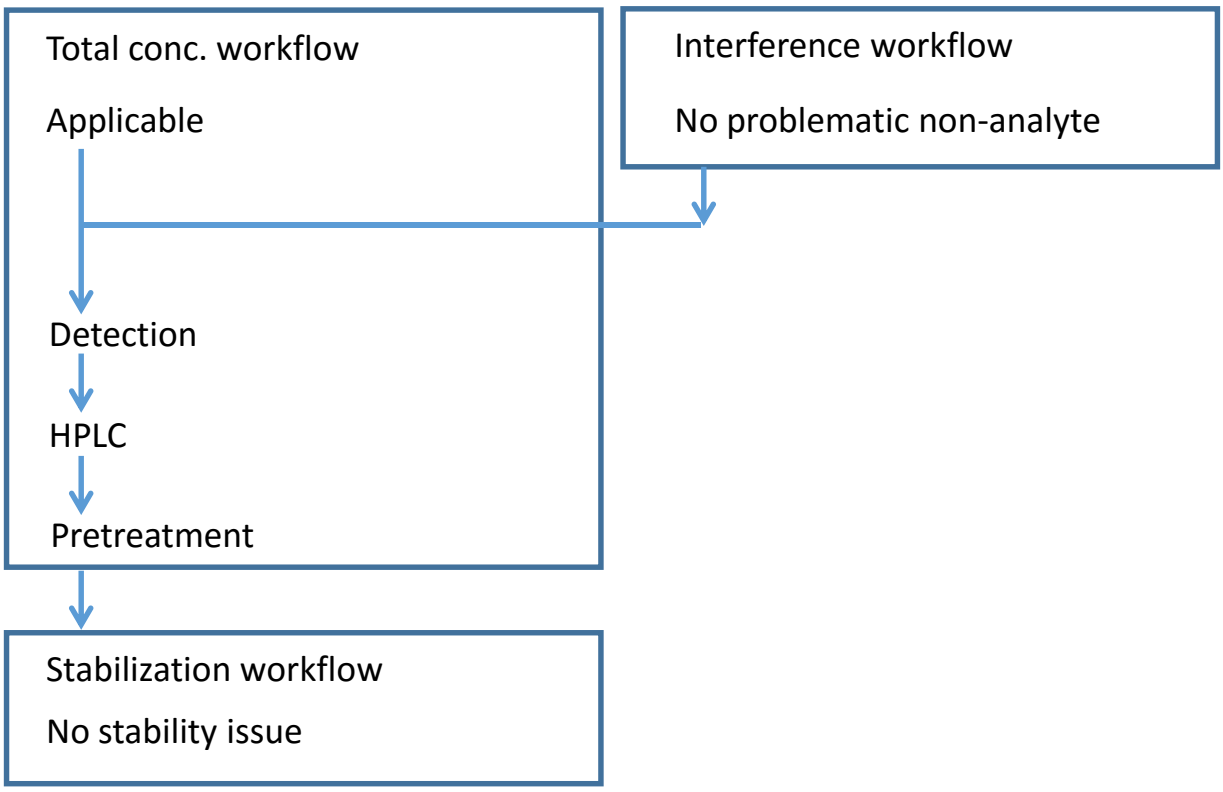

Fig. 2-13. Overview of method development of ASP2151.

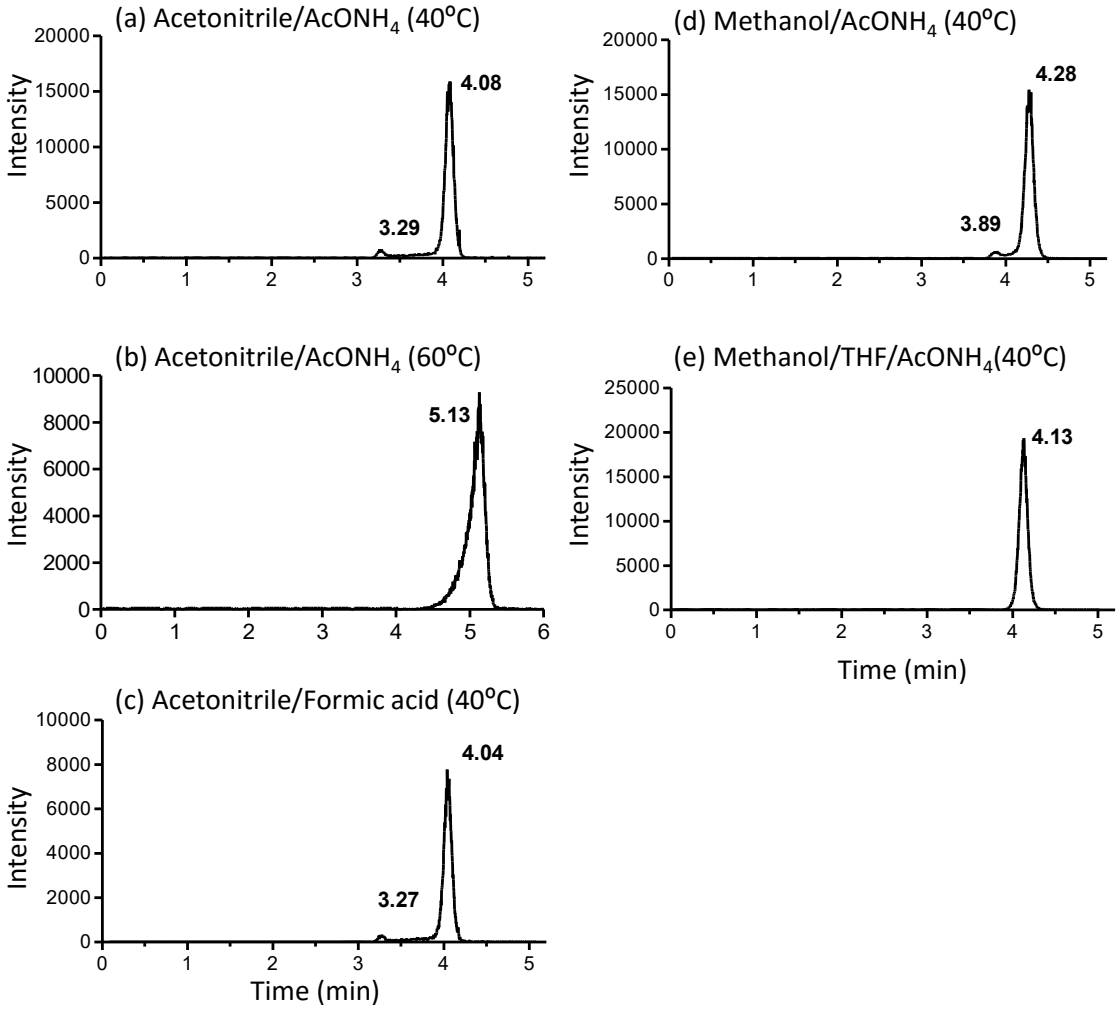

Fig. 2-14. Mass chromatograms of ASP2151 standard solutions using various HPLC mobile phases and column temperatures. (a) Acetonitrile/20 mmol/L ammonium acetate (40:60, v/v) at 40°C, (b) acetonitrile/20 mmol/L ammonium acetate (35:65, v/v) at 60°C, (c) acetonitrile/0.1% formic acid (40:60, v/v) at 40°C, (d) methanol/20 mmol/L ammonium acetate (55:45, v/v) at 40°C, (e) methanol/THF/20 mmol/L ammonium acetate (45:5:50, v/v/v) at 40°C. $AcONH_4$, ammonium acetate.

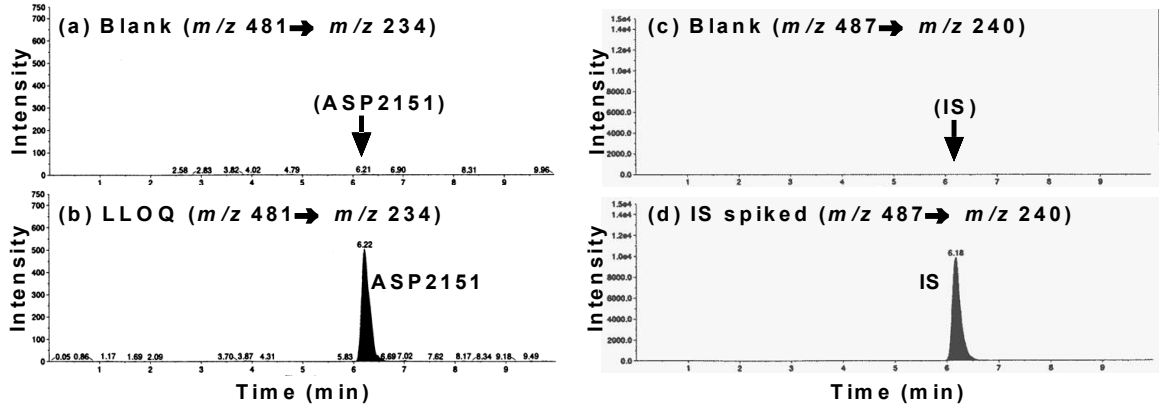

Fig. 2-15. Representative mass chromatograms of (a) blank dog plasma monitored for *m/z* 481→234, (b) blank dog plasma spiked with ASP2151 (5 ng/mL; LLOQ) for *m/z* 481→234, (c) blank dog plasma for *m/z* 487→240, (d) blank dog plasma spiked with IS for *m/z* 487→240.

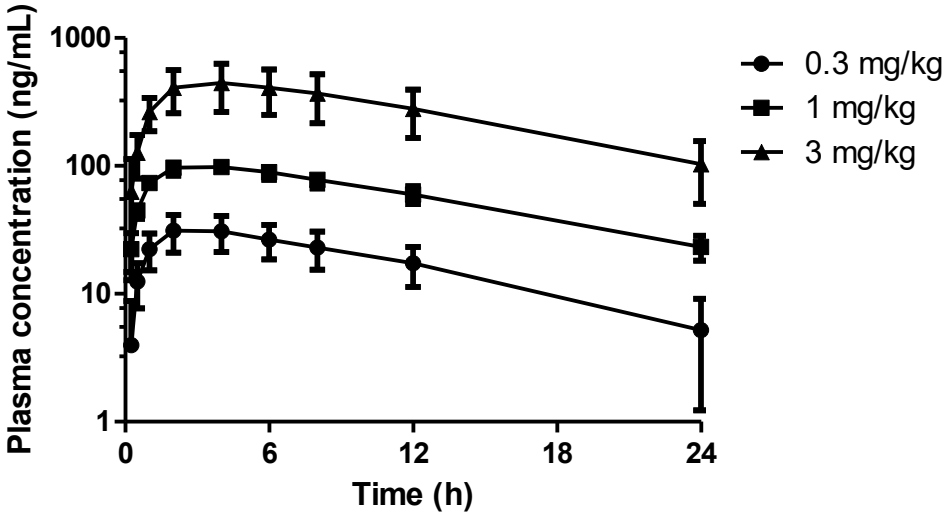

Fig. 2-16. Plasma concentrations in dogs after a single oral administration of ASP2151 (0.3, 1, and 3 mg/kg) (mean ± standard deviation, n=4).

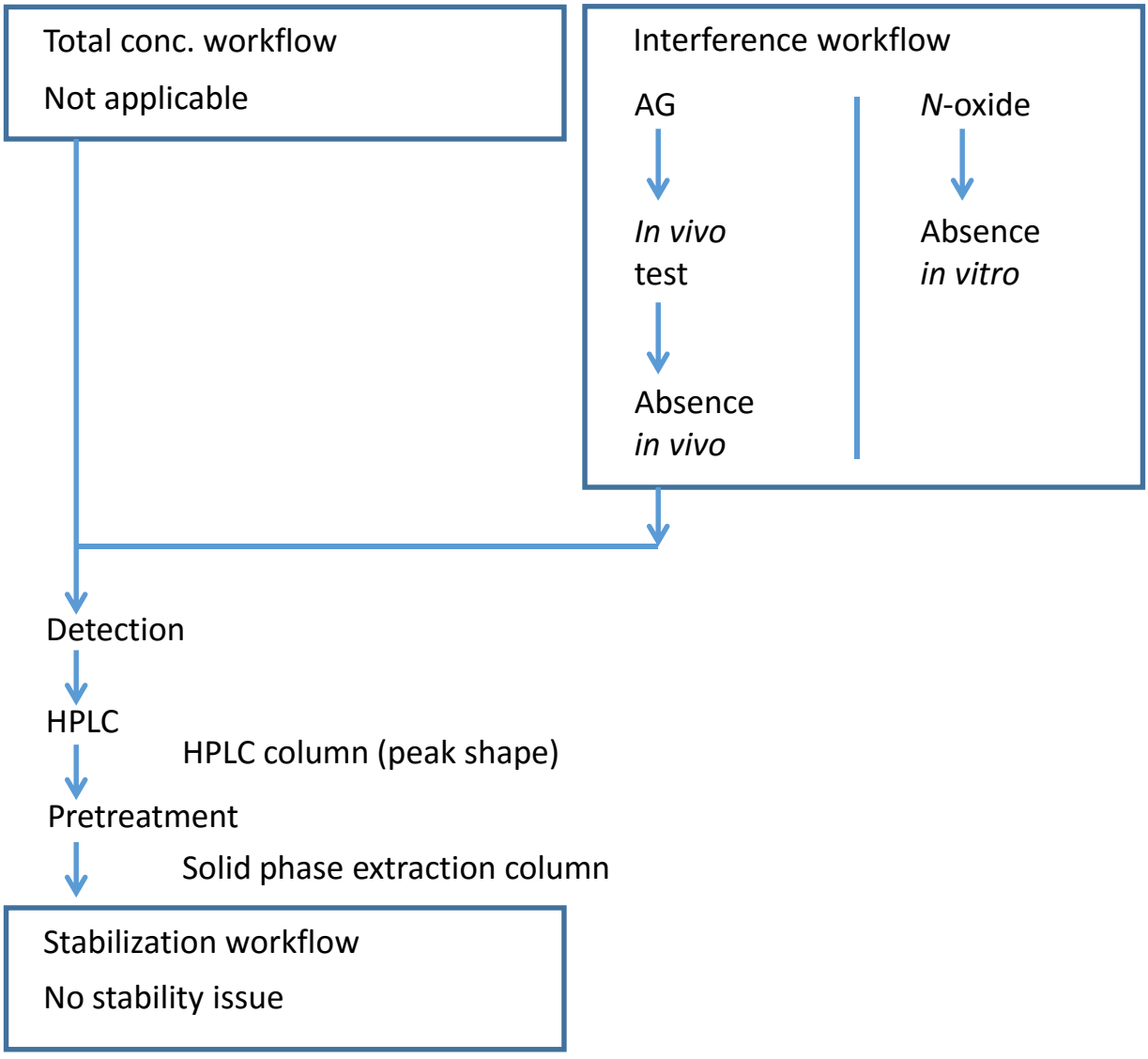

Fig. 2-17. Overview of method development of ASP3258.

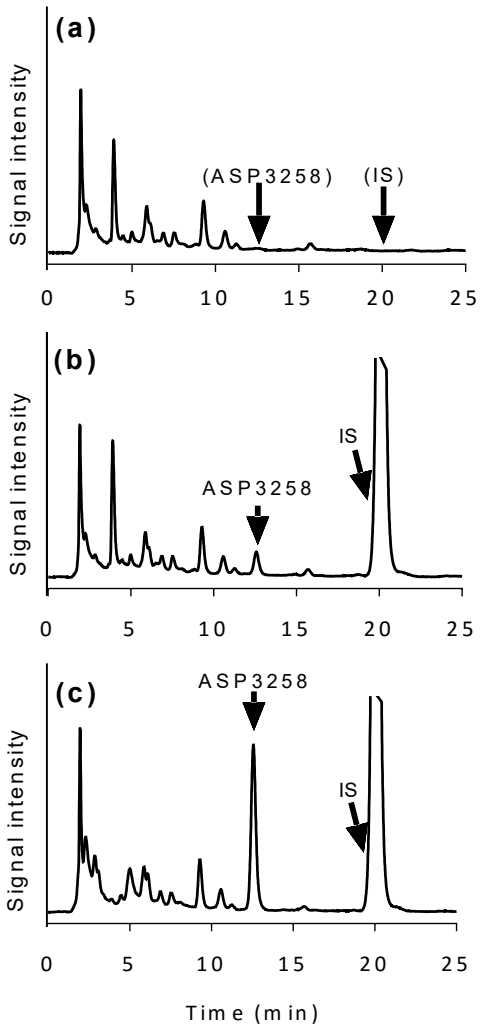

Fig. 2-18. Representative HPLC chromatograms of (a) blank rat plasma, (b) blank rat plasma spiked with ASP3258 (2.5 ng/mL) and IS, and (c) a rat plasma sample obtained at 8 h after a single i.v. administration of ASP3258 at 0.1 mg/kg.

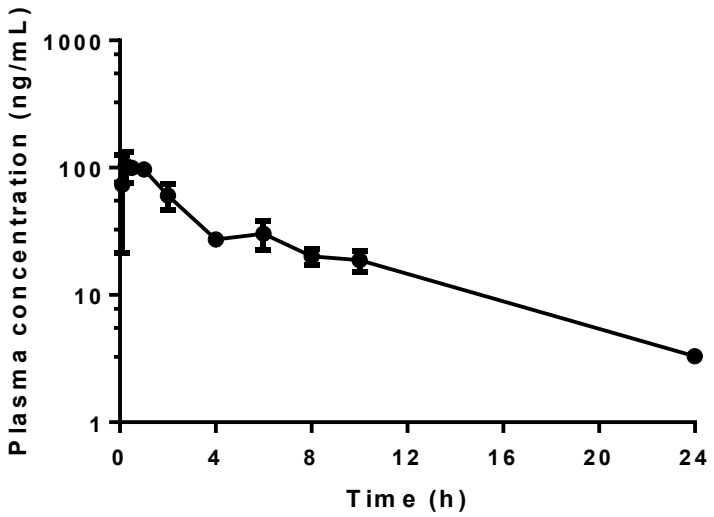

Fig. 2-19. Plasma concentrations in rats after a single i.v. administration of ASP3258 (0.1 mg/kg) (mean ± standard deviation, n=3).

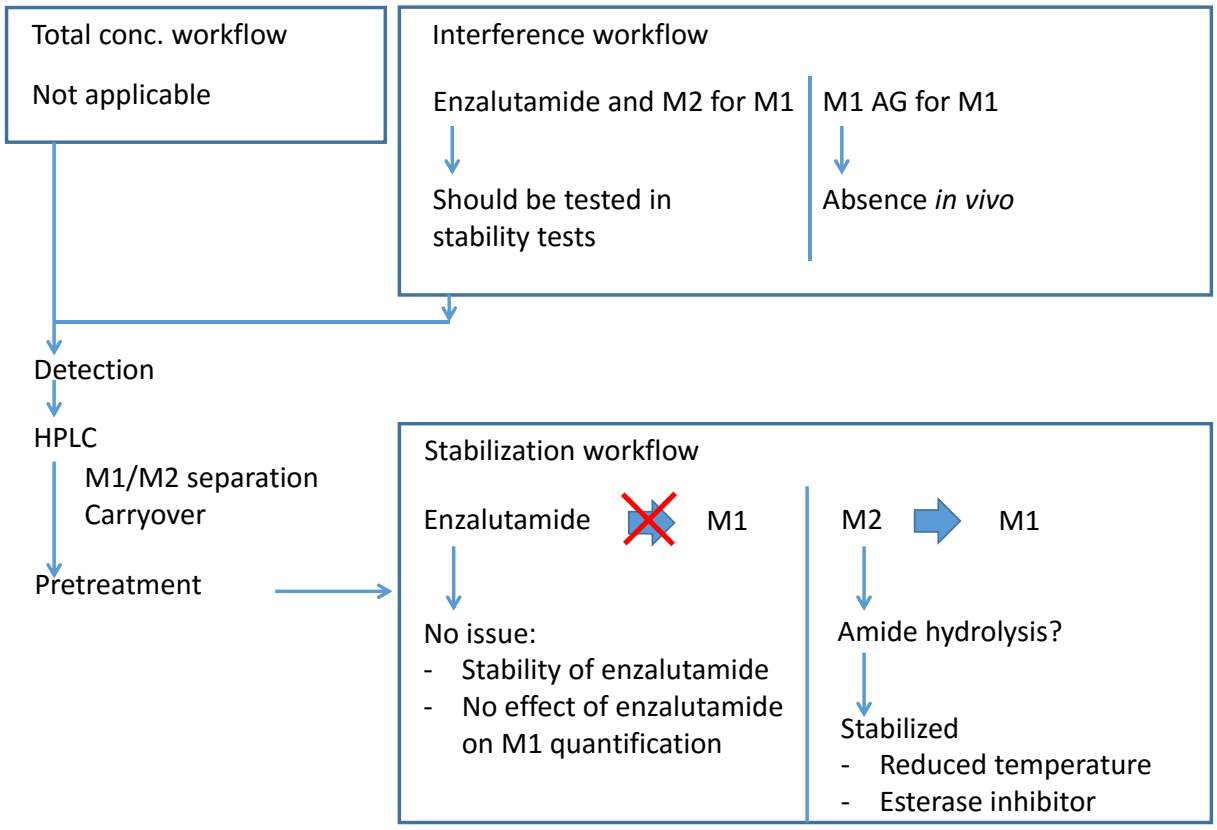

Fig. 2-20. Overview of method development of enzalutamide, M1, and M2.

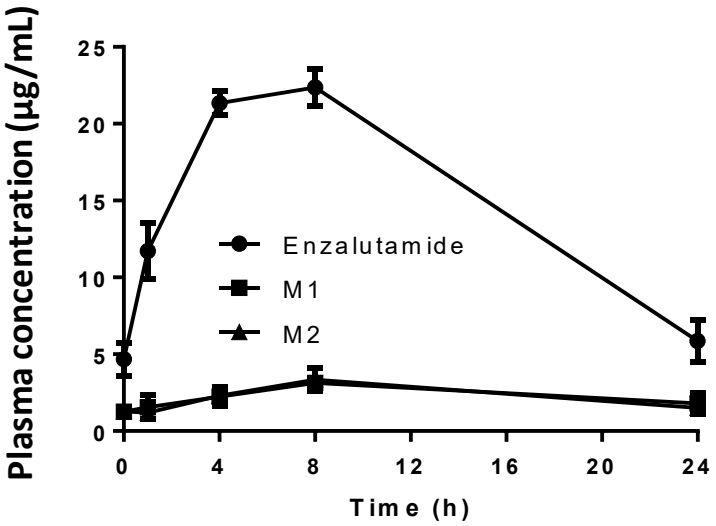

Fig. 2-21. Plasma concentrations in male mice on Day 28 after repeated oral administration of enzalutamide at 60 mg/kg/day. (mean ± standard deviation, n=3). Plots overlapped, as M1 and M2 concentrations were similar.

CHAPTER 3 CONCLUSION

3.1 General discussion

In this research, workflows were established with a view to three issues in the development of methods for the determination of chemosynthetic drugs and their metabolites in animal biological fluids. The workflows were applied to the development of methods for three drug candidates - ASP2151, ASP3258, and enzalutamide - and the usefulness of the workflows were evaluated through method validation and sample analysis. In addition, the pharmacokinetics of ASP2151, ASP3258, and enzalutamide were assessed in sample analysis.

Method development for ASP2151 proceeded well using the total concentration workflow. This demonstrated that the established workflow can support efficient development of highly reproducible methods for total concentration of conformers. Although ASP2151 did not form a single peak in the first and second steps of HPLC method examination, but finally did at the third step, the author believes it is not reasonable to change the order of the steps in the workflow, as this is the first case in which organic modifiers played a key role in conformer analysis.

To the author's knowledge, the total concentration workflow is the first general workflow for the method development of conformers, and makes it feasible for the pharmaceutical industry to develop the methods. Clayden et al. wrote that it was difficult to develop a compound possessing conformers and suggested that one of the solutions would be to modify the chemical structure in an early drug discovery stage (Clayden et al., 2009). However, it is noteworthy that important pharmaceutical products have been discovered from compounds possessing conformers. Enalaprilat (Bouabdallah et al., 2003) and cyclosporine (Bowers and Mathews, 1985) are good examples. The present research can contribute to medical care by facilitating the development of conformer-associated drug candidates that had been set aside in the past.

With regard to an issue derived from the conversion of non-analytes, it is important to manage the interference risk without inefficient use of time and manpower resources. Thus, the interference management workflow has to provide a comprehensive list of convertible non-analytes, and its recommended tasks should be sufficiently easy considering the extent of the risk. In the present research, the interference management workflow successfully detected risks associated with the quantification of ASP3258 and enzalutamide metabolite M1, and in the opinion of the author, the resources required for this were reasonable. Further, the workflow predicted that quantification of ASP2151, enzalutamide, and its metabolite M2 was not interfered with by non-analytes, and thus required no tasks. As the sample analysis with these analytes proceeded smoothly, the list of convertible non-analytes in the workflow was confirmed to be comprehensive.

With regard to an issue concerning analyte stabilization, a decrease in concentration was not observed with ASP2151, ASP3258, enzalutamide, and its metabolite M1. The enzalutamide metabolite M2 showed a concentration decrease in intact plasma, with an increase in M1 concentration. The analyte stabilization workflow was able to deal with this issue effectively by the sequential use of cooling and an esterase inhibitor.

The three workflows were constructed to eliminate the need for unnecessary experiments associated with conversion issues and to streamline the necessary tasks. The author expects that laboratory researchers can use the workflows in their daily work and achieve substantial savings in labor and time. As drug development is promoted using limited resources, the author believes that the labor and time saving achievable with the workflows considerably promotes drug development.

Drug concentration data in biological fluids play an important role in evaluating the safety and efficacy of drug candidates. If drug concentration data are not reproducible or accurate, the profile of the drug candidate can be misunderstood, and development of the drug

can be set in the wrong direction. In addition, if the pharmaceutical company does not find the error until it is pointed out by the regulatory agency at the time of new drug application, the new drug application must be rejected. In this context, it is considered that the workflows constructed in the present research will greatly contribute to the development of drugs, through delivery of accurate and reproducible data on drug concentrations.

As ASP3258 and ASP2151 are new compounds, their absorption and plasma concentrations have not been publicly reported. As for enzalutamide, two articles written by the author and colleagues reported clinical pharmacokinetics (Gibbons et al., 2015a, 2015b). However, animal pharmacokinetics data are not available at all. Therefore, the data obtained in the present research are valuable.

It was revealed that ASP3258, ASP2151, and enzalutamide are eliminated gradually from the body. In addition, it was shown that oral absorption of ASP2151 and enzalutamide are relatively rapid and that their plasma concentrations increase with an increase in the oral doses. These data suggest that these three compounds have excellent properties as drug candidates from the point of view of pharmacokinetics.

A future perspective of the present research is as follows. The analytes and methodologies in the present research differ from each other markedly. The analytes have different chemical structures. In addition, two detection methodologies were used in the present research, MS/MS and fluorescence. Further, one-analyte methods and a multiple analyte method were developed. As the workflows were evaluated with these very different analytes and methodologies, the author believes that the evaluation of these workflows is valuable. Nonetheless, application of these three workflows to a wider range of analytes and evaluation of the workflows are important future tasks. This is especially true for the total concentration workflow and analyte stabilization workflow, as these two workflows were used essentially for only one analyte (ASP2151 and M2, respectively).

Another future direction is to extend the scope of the present research to clinical studies. The total concentration workflow is likely to work well without modification, given that the workflow is unlikely to be affected by the differences between animals and humans. The analyte stabilization workflow can be applied to clinical studies after minor modifications in consideration of the fact that clinical sites face difficulties in using some stabilization chemicals and adding accurate amounts of stabilizers (Hilhorst et al., 2015). On the other hand, the interference management workflow is likely to require essential adjustment, as *in vivo* human samples are not available during method development and the workflow includes the use of *in vivo* samples. The uncertainties derived from the absence of *in vivo* human samples may be overcome by the use of *in vivo* drug metabolism studies in animals and *in vitro* drug metabolism studies using human specimens.

Also, the three workflows established in the present research can be more sophisticated. The present research recommends that all of the three workflows should be used one by one when a new bioanalytical method is developed. This approach might sound tedious for laboratory researchers, especially if they face multiple issues, not only compound conversion issues but also other issues. If the three workflows (and potentially workflows which are out of scope of the present research) are combined into one holistic workflow, accurate and precise bioanalytical methods can be developed more efficiently. A new theory or experimental data about compound conversion issues and their solutions may facilitate this refinement process.

3.2 General conclusion

In the present research, three workflows were constructed for the development of bioanalytical methods for drugs associated with issues derived from the conversion of compounds and were applied to three drug candidates, namely ASP2151, ASP3258, and

enzalutamide. The results of method validation and sample analysis using the developed methods demonstrated that the workflows are actually useful in the development of bioanalytical methods for drugs with conversion issues. In addition, sample analysis using the developed methods clarified the pharmacokinetics of ASP2151, ASP3258, and enzalutamide, and could facilitate the development of these drug candidate compounds.

NOTICE

This thesis was based on the following research articles.

[1] Ohtsu, Y., Otsuka, S., Nakamura, T., Noguchi, K., 2015. Regulated bioanalysis of conformers - A case study with ASP2151 in dog plasma and urine. J. Chromatogr. B 997, 56–63. doi:10.1016/j.jchromb.2015.05.028

[2] Ohtsu, Y., Takanuki, F., Fukunaga, Y., Noguchi, K., 2015. Determination of ASP3258, a novel phosphodiesterase type 4 inhibitor, in rat plasma by high-performance liquid chromatography with fluorescence detection and its application to pharmacokinetic study. Biomed. Chromatogr. 29, 161–163. doi:10.1002/bmc.3262

[3] Ohtsu, Y., Thakker, D.R., Gibbons, J.A., Tsubota, K., Otsuka, S., Arai, H., 2015. Determination of the androgen receptor inhibitor enzalutamide and its metabolites in animal plasma and brain homogenates using LC-MS/MS and its application to pharmacokinetic studies. Chromatography 36, 115–122. doi:10.15583/jpchrom.2015.029

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Anything the author expresses in this thesis is the author’s personal opinion and does not necessarily represent positions, strategies, or opinions of a company or organization the author works with or belongs to. The co-authors of the above-mentioned articles take responsibility for each article but not for this thesis.

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