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Development and implementation of a stereoselective normal-phase liquid chromatography–tandem mass spectrometry method for the determination of intrinsic metabolic clearance in human liver microsomes☆

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ABSTRACT

The stereoselective determination of stereoisomers in biological samples provides vital information on stereospecific metabolism and pharmacokinetic profiles of the drugs. Despite the unique advantage and the great success of normal-phase (NP) HPLC for the separations of drug stereoisomers using polysaccharide-type chiral stationary phases (CSPs), the technique is rarely applied to quantitative HPLC–MS–MS bioanalysis. This is, at least in part, due to the incompatibility between the usual mobile phase (*n*-hexane or *n*-heptane) in normal-phase HPLC and the MS ionization sources which poses a potential detonation hazard. An environmentally friendly and nonflammable alternative solvent, ethoxynonafluorobutane (ENFB), was reported previously to potentially provide an ideal solution for combining the powers of stereoselective NP chromatographic separation and MS–MS detection. In this study, a stereoselective NP-HPLC–MS–MS method was developed using ENFB to quantify a pair of Bristol Myers Squibb (BMS) proprietary drug stereoisomers and their ketone metabolite for an *in vitro* study, which demonstrated, for the first time, the practical applicability and utility of ENFB for bioanalysis in pharmaceutical industry. The effects of different organic modifiers and temperature, as well as the comparison between ENFB and the usual solvent, heptane, for the separation, are discussed. The resolution of the stereoisomers was achieved using 63% of 3:1 mixture of ethanol and methanol with 37% ENFB on a Chiralpak AD-H column at 50 °C. High sensitivity was obtained using the MS–MS detection in the positive ion atmospheric pressure chemical ionization (APCI) mode. The lower limit of quantitation (LLOQ) for the first stereoisomer and the ketone metabolite was 5 ng/mL, and was 10 ng/mL for the second isomer in the human liver microsome–potassium phosphate buffer matrix. The linear dynamic range of 5–1000 ng/mL for both isomers and 10–1000 ng/mL for the metabolite were demonstrated with $R^2 \geq 0.997$. The precision of the analysis was <5% R.S.D. at or above the nominal concentration of 80 ng/mL, and <20% R.S.D. at 8 ng/mL. The mean bias was less than 15%. Extraction recovery and acceptable matrix interference were demonstrated using one isomer and the ketone, and better than 75% recovery and less than 25% ion suppression or interference were found. The method was successfully implemented for an *in vitro* intrinsic metabolic clearance study.

1. Introduction

Living organisms are based on a plethora of chiral molecules and often display different biological responses to drug enantiomers. It is not uncommon for one drug enantiomer to be active while the other is toxic in biological systems [1,2]. Thus, the US Food and Drug Administration (FDA) has required evaluation of each enantiomer in developing stereoisomeric drugs [3]. As a result, the pharmaceutical industry has increased its emphasis on the synthesis, isolation and analysis of enantiomerically pure drugs. The distribution of

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worldwide approved drugs from 1983 to 2002 and FDA approved drugs from 1991 to 2002 indicates that single-enantiomers surpassed achirals whereas racemic drugs represented the minority category [4]. In 2003, nine out of ten top-selling drugs (accounting for approximately 50 billion US$) were chiral molecules, and six of these were sold as single-enantiomer drugs [5].

The strong focus on the development of single-enantiomer drugs has stimulated a considerable interest in the stereospecific pharmacokinetics, metabolism, toxicology and clinical pharmacology of chiral drug molecules. Chiral inversion may occur either due to chemical instability or biological catalysis resulting in different stereoselective biological activities and pharmacokinetic profiles [6]. Furthermore, drug metabolism involves a high degree of stereoselectivity in drug disposition. For example, stereoselective induction or inhibition of enzymes will affect metabolism of both enantiomers, but possibly to a different extent [8]. That is, each enantiomer of a chiral drug can be metabolized at different rates by the same enzymes [7] and enantiomers of some chiral drugs may be metabolized by different enzymes [9]. Therefore, the stereoselective determination of the enantiomers in *in vitro* and *in vivo* studies can provide vital information to improve pharmacokinetic and safety properties of the drug, and is of potential clinical importance. Development of highly efficient chromatographic methods for bioanalysis of low levels of each stereoisomer is a necessity in the pharmaceutical industry to search for drugs with greater therapeutic benefits and low toxicity. The objective of this work was to develop a stereoselective HPLC–MS–MS method for the analysis of two BMS proprietary drug stereoisomers and their potential ketone metabolite in human liver microsomes to examine the stereospecific *in vitro* intrinsic metabolic clearance.

Major developments in chiral stationary phases have led to dramatic advances in stereoselective chromatography in the past few decades [10]. High-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) on chiral stationary phases (CSPs) have been the most widely utilized stereoselective separation techniques in pharmaceutical industry [11–13]. The derivatized polysaccharide CSPs first developed by Okamoto et al. [14] and trademarked as Chiralcel and Chiralpak by Daicel Chemical Industries are the most widely used CSPs due to their broad stereoselectivity and versatility. While the derivatized polysaccharides are multi-modal CSPs, normal-phase HPLC or SFC is the preferred mode of separation in the context of obtaining pure enantiomers, especially in a preparative application, since the solvents can be removed relatively quickly [15]. Moreover, normal-phase HPLC and SFC were found to be generally superior for chiral resolution due to improved stereospecific molecular recognition characteristics [16,17]. According to Francotte and Lindner, "under reversed-phase conditions the chiral recognition performance of polysaccharide-type CSPs is often diminished compared to normal-phase conditions involved in chiral recognition" [18]. On a Chiralpak AD column, used in this study, the dimethylphenylcarbamate provides hydrogen-bonding, dipole–dipole and π–π stacking sites, and the conformation of the amylose provides steric interactions. Since the hydrogen-bonding, dipole–dipole and π–π interactions are largely enhanced in the low polar solvents, normal-phase chromatography has been the most widely utilized mode of chromatography for chiral resolution.

The vast majority of stereoselective separations have been accomplished using HPLC with ultraviolet (UV) detection [19]. Despite the simplicity and reliability of UV detection, it usually lacks the sensitivity and specificity necessary for the analysis at low concentrations in complex biological matrices. The high selectivity and sensitivity of tandem mass spectrometry (MS–MS) combined with HPLC provides superior detection limits relative to LC–UV for the analysis of small molecules in biological samples. Reversed-phase (RP) LC methods dominate HPLC–MS–MS analyses including chiral separations. This is due, at least in part, to the potential explosion hazard associated with the use of typical normal-phase (NP) solvents (*n*-hexane or *n*-heptane) in the MS ionization sources.

In 2001, Kagan [20] suggested the use of ethoxynonafluorobutane (ENFB), an environmentally friendly, fluorinated solvent, as a replacement of *n*-hexane or *n*-heptane for NP HPLC separation. Kagan et al. [21] also demonstrated the compatibility of ENFB for LC–APCI–MS, and reported that APCI response in ENFB mobile phases is stronger for non-polar compounds and comparable for polar compounds comparing to RP mobile phase systems using ESI. However, this approach has not yet been widely utilized, and no NP-HPLC–MS–MS application using ENFB has been reported for bioanalysis to the best of our knowledge.

We describe, herein, the development of a stereoselective NP-HPLC method substituting ENFB for *n*-heptane. The method was successful implemented for NP-HPLC–APCI–MS–MS analysis of a pair of BMS proprietary drug stereoisomers and their potential metabolite in human liver microsomes. The intrinsic metabolic clearance of each of stereoisomer and their potential chiral conversion were examined in the *in vitro* study.

2. Experimental

2.1. Reagents and chemicals

The stereoisomers were proprietary drug molecules prepared at Bristol Myers Squibb Co. (BMS), with a chiral center on a 2-hydroxypropyl functional group. The stereoisomers are called BMS-Isomer-A and BMS-Isomer-B in this paper. The metabolite was the corresponding ketone from the hydroxyl group. Ethoxynonafluorobutane was purchased from Sigma–Aldrich (St. Louis, MO). Ethyl alcohol (200-proof ACS/USP Grade) was purchased from Pharmco (Brookfield, CT). Methanol and isopropanol, both HPLC grade, were purchased from J.T. Baker (Phillipsburg, NJ). Acetonitrile, HPLC grade, was purchased from Burdick & Jackson (Muskegon, MI). The internal standard was a related proprietary BMS compound, and will called BMS-IS in this paper. The stock solutions were prepared at a concentration of 1 mg/mL in 100% methanol. The concentration of the Internal Standard was 100 ng/mL in acetonitrile.

2.2. HPLC and MS instrumentation

A Thermo Finnigan Quantum Ultra (San Jose, CA) equipped with an APCI source was used for tandem mass spectrometry (MS–MS). MS–MS analysis was performed using selected reaction monitoring (SRM) and positive ionization for all compounds. The chromatographic system consisted of Shimadzu LC-10AD VP liquid chromatographic pumps, Shimadzu SCL-10A VP system controller, DGU 14A Degasser (Columbia, MD), Thermo Electron Hot Pocket column oven (San Jose, CA), and CTC Analytics PAL autosampler with a refrigerated plate compartment (set at 4 °C) from Leap Technologies (Carrboro, NC). The chiral separation was first developed and optimized using a Shimadzu HPLC with UV detection (PDA) and an Agilent 1100 HPLC–DAD–APCI–MSD system.

2.3. Columns, mobile phases and ionization conditions

Separation of BMS-Isomer-A, BMS-Isomer-B, their ketone metabolite, and BMS-IS was carried out on a 4.6 mm × 250 mm 5 μm Chiralpak AD-H column (Chiral Technologies) at a flow rate of 0.9 mL/min. The final optimized mobile phase consisted of 63% of 3:1 ethanol:methanol and 37% ethoxynonafluorobutane. The column temperature was kept at 50 °C. Ionization parameters were optimized by infusing the compounds with a syringe pump into

the APCI source of the mass spectrometer and resulted in the following: discharge current of 4.0 kV, vaporizer temperature 350 °C, sheath gas pressure 15 (arbitrary units), auxiliary gas pressure 20 (arbitrary units), capillary temperature 350 °C, and collision pressure of 1.3 mTorr. The distinct SRM transitions, for BMS-Isomer-A, BMS-Isomer-B, the ketone and the BMS-IS, were monitored.

2.4. Metabolic incubations and sample preparation

The *in vitro* samples were generated in human liver microsomal (HLM) incubations for each stereoisomer in duplicate. Incubation mixtures (100 μL total volume) consisted of human liver microsomes (1 mg microsomal protein/mL), a single stereoisomer as the substrate (5.0 μM), and 1 mM NADPH in 50 mM potassium phosphate buffer, pH 7.4. Reactions were started with the addition of NADPH and shaken in an open air water bath at 37 °C. At $T = 0$, 15, 30 and 60 min, 100 μL of an ice-cold solution containing 100 ng/mL of internal standard in acetonitrile was added to each mixture. All samples were placed on ice for 15 min. The samples were then centrifuged at 3000 rpm at 4 °C for 30 min to remove precipitated proteins. 100 μL of the supernatant from each sample was transferred to another plate and diluted with an additional 150 μL of 100 ng/mL internal standard in acetonitrile. Controls without NADPH were also taken at 0 and 60 min.

For the quantification, a 100 μg/mL mixture containing all three compounds (the stereoisomers and the ketone metabolite) in acetonitrile was prepared from 1 mg/mL stock solutions. Working stock solutions at concentrations of 10, 1, and 0.1 μg/mL were prepared by serial dilution from the 100 μg/mL stock. Calibration standards at concentrations of 1, 2, 5, 10, 50, 250, 500, 1000, 2000, 5000 ng/mL were prepared by standard addition from the working stock solutions into an HLM/0.05 M phosphate buffer matrix. Quality control (QC) samples at concentrations of 800, 80, 8 ng/mL of each compound were prepared in triplicate in the same manner as the calibration standards. A 50 μL volume of each standard or QC sample was transferred into a 96 well plate. A 200 μL volume of the 100 ng/mL internal standard in acetonitrile was added to each well for protein precipitation. The samples were vortex mixed for 2 min, centrifuged for 1 min and evaporated to dryness under nitrogen at 50 °C. The samples were reconstituted with the mobile phase. Samples were vortex mixed and transferred to an autosampler plate. A volume of 5 μL was injected into the NP-HPLC/MS–MS system.

2.5. Method qualification

Since the purpose of the analysis was for an *in vitro* screening read-out in an early chemotype exploratory research, and the amount of the material was also very limited, full validation was not required, and only partial validation experiments were performed to qualify the method. The method was qualified by analysis of the quality control samples prepared as described above.

Intra-assay reproducibility was performed by preparing one standard curve and six sets of the QC samples. Precision was expressed as the relative standard deviation. The assay was completed within the same day, and inter-day reproducibility was not conducted. Accuracy was measured according to the following equation: percent difference (bias, %) from nominal value = $(X - \mathrm{NC})/\mathrm{NC} \times 100$, where X is the measured mean value of QC samples and NC is the nominal concentration.

Sample stability of all three compounds in autosampler (20 °C) was verified by injecting the QC samples at beginning and at the end of the run over a period of 24 h covering the length of the analysis. The retention times and peak areas were compared.

Extraction recovery was checked in 12 replicates by adding BMS-Isomer-A and the ketone at 500 ng/mL to the incubation mixture and extracting with acetonitrile as described above except the internal standard was added to the supernatant. The peak area ratios of the compounds to internal standard were compared with blank

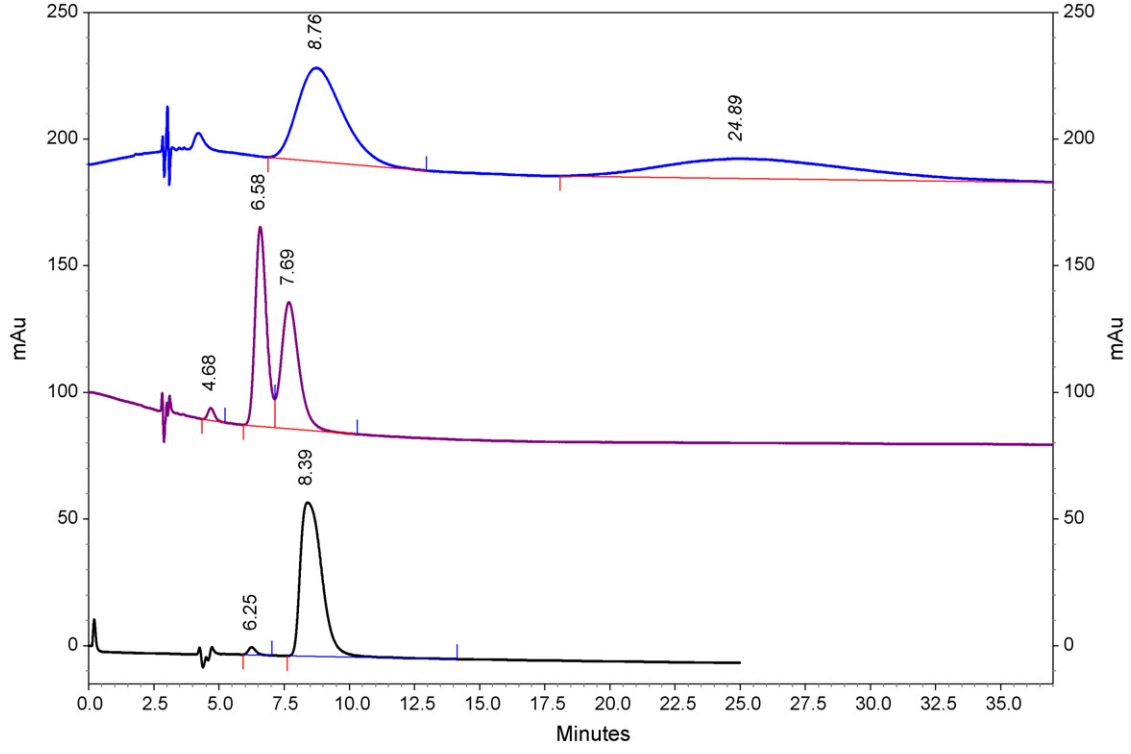

Fig. 1. Effect of alcohol types on the stereoselective separation: Upper profile: 30% ENFB/70% methanol at 1 mL/min; Middle profile: 30% ENFB/70% ethanol at 1 mL/min; Bottom profile: 30% ENFB/70% isopropanol at 0.65 mL/min. Column temperature: 40 °C.

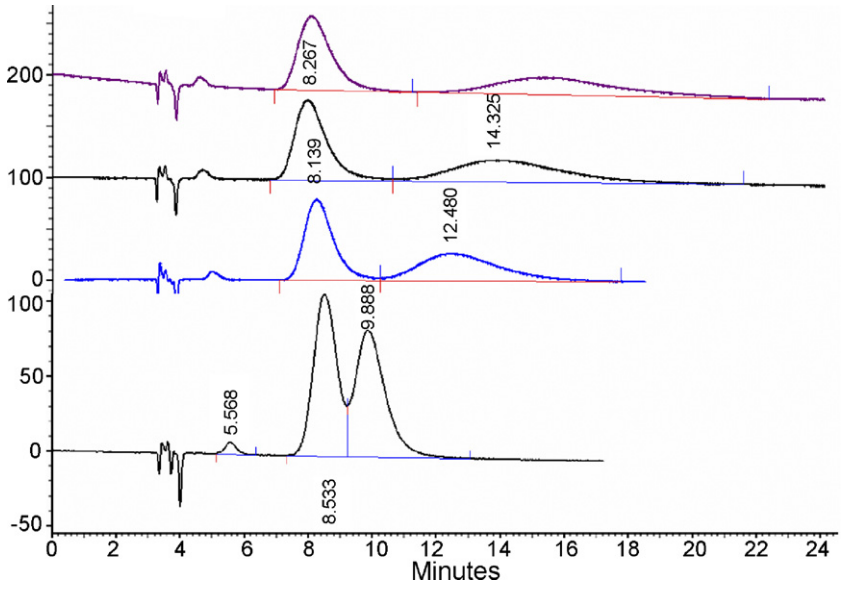

Fig. 2. Effect of the alcohol ratio on the resolution: 55% heptane and 45% alcohol mixture at the different ratio; column at 25 °C. Top Profile: 3/5 EtOH/MeOH; Upper Profile: 1/1 EtOH/MeOH; Lower Profile: 2/1 EtOH/MeOH; Bottom Profile: 1/0 EtOH/MeOH.

samples (extracted with acetonitrile as above and followed by spiking same amounts of the compounds and internal standard into the supernatant).

Matrix interferences were also checked at 500 ng/mL by comparing (dividing) the peak area ratio of the sample spiked with the compounds and internal standard after extraction with that of the sample prepared in water. The average ratio of 12 replicates was calculated.

3. Results and discussion

The stereoisomers, BMS-Isomer-A and BMS-Isomer-B, were proprietary drug molecules of Bristol Myers Squibb Co. and contained a chiral center on a 2-hydroxypropyl functional group. The potential metabolite of the isomers was the corresponding ketone from the hydroxyl group. A sensitive method for simultaneous determination of both stereoisomers and the metabolite was

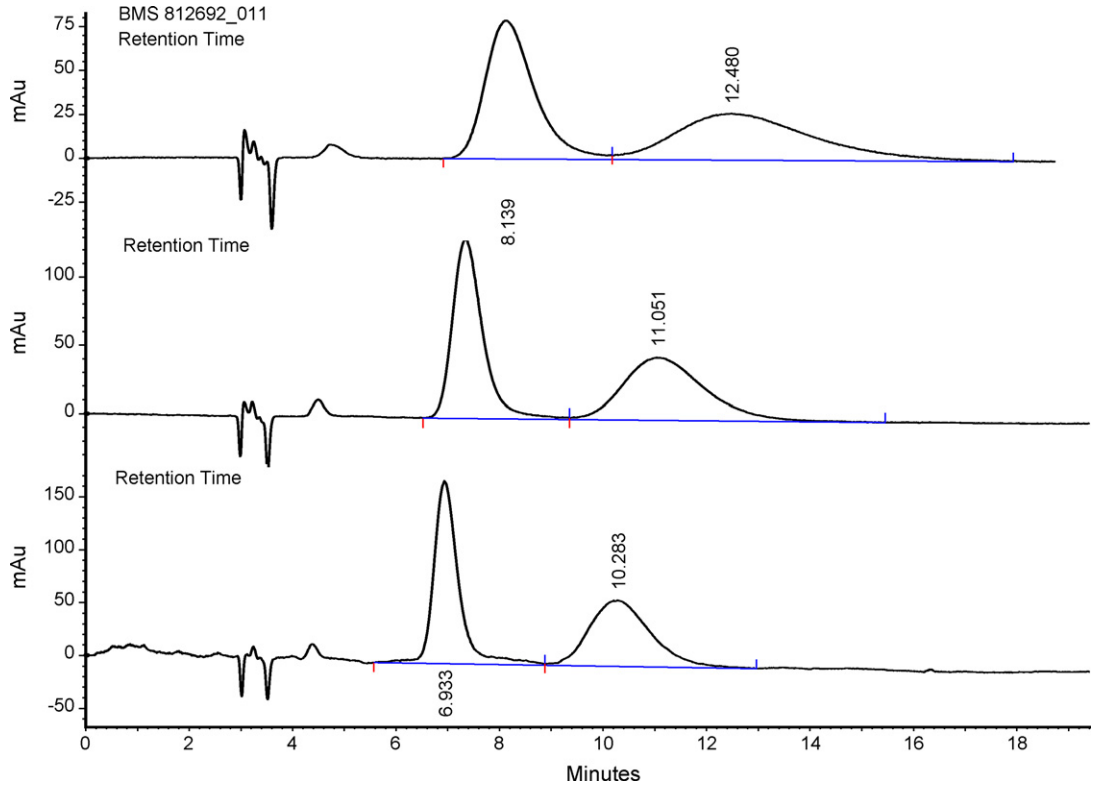

Fig. 3. Effect of column temperature on the separation. 55% Heptane/45% of 1:1 MeOH:EtOH. Profiles from top to bottom: 25 °C vs. 35 °C vs. 40 °C.

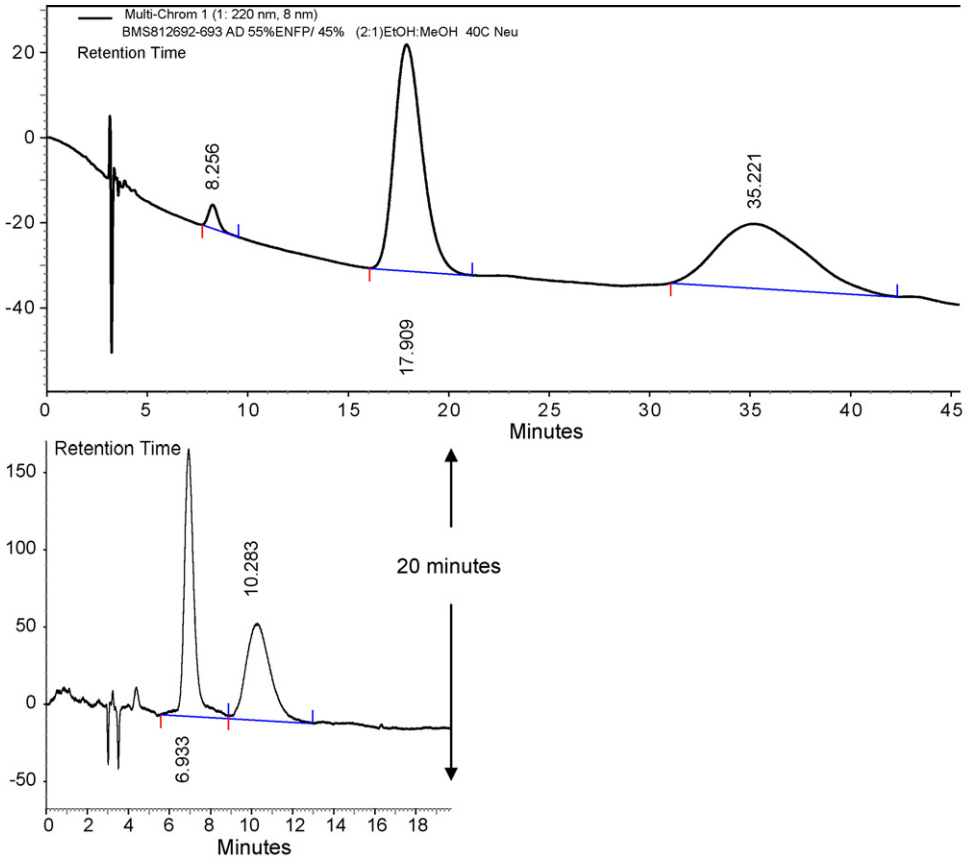

Fig. 4. Comparison of ethoxynonafluoropentane (top profile) and heptane (bottom profile) with 45% 1:1 MeOH:EtOH at 40 °C column temperature.

required to examine the chiral inversion and stereospecific intrinsic metabolisms of both stereoisomers.

3.1. Chiral NP-HPLC method development

Chirobiotic R and V, Chiralpak AD-R, OD-R, OJ-R, AS-H and a Cyclobond I RSP CSPs were screened under RP-HPLC conditions, and AD-H, OD-H, OJ-H and AS-H were screened under NP-HPLC conditions for the resolution of the analytes. Only the Chiralpak AD-H column under normal-phase conditions showed a promise for the stereoselective separation. Higher success in normal-phase than reversed-phase stereoselective chromatography is not uncommon for polysaccharide-type CSPs. The low polarity of the normal-phase media (consist of apolar organic solvents such as heptane with polar modifiers such as alcohol) is capable of stabilizing polar and dipolar interactions and disrupting hydrophobic interactions [18]. As a result, it creates ideal environments for polysaccharide-type CSPs where the stereoselective molecular interactions, namely hydrogen-bonding, dipole–dipole and π–π stacking interactions, can be maximized.

In NP-HPLC or SFC, varying the type or concentration of the alcohol modifier often results in different enantioselectivity and efficiency on derivatized polysaccharides CSPs. More often branched and/or long chain alcohols tend to provide higher selectivity and short, linear alcohols show higher efficiencies [11]. However, for the stereoisomers in this study, the stereoselectivity increased as peak broadened (efficiency decreased) in the order of isopropanol > ethanol > methanol when used as the organic additive in the mobile phase. Since methanol is not miscible with heptane or hexane, ethoxynonafluorobutane (ENFB) was used for the comparison of the three alcohols. As clearly shown in Fig. 1, methanol provided enantioselectivity, but also resulted in significantly broadened peaks. Ethanol sharpened the peaks while maintaining partial resolution. Isopropanol destroyed the stereoselectivity.

Table 1
Intra-day assay precision and accuracy

Analyte	Nominal concentration (ng/mL)	Predicted concentration (ng/mL) (mean ± S.D., $n = 6$)	Precision (%R.S.D.)	%Bias
"BMS-Isomer-A"	8	7 ± 1	15.6	−12.8
	80	74 ± 3	4.6	−8.0
	800	855 ± 36	4.2	5.2
"BMS-Isomer-B"	8	7 ± 1	14.3	−6.6
	80	73 ± 2	3.2	−8.4
	800	864 ± 37	4.2	8.0
"Ketone"	80	69 ± 3	4.3	−14.2
	800	852 ± 58	6.8	6.6

Since ethoxynonafluorobutane (ENFB) provides similar selectivity as heptane [22], we used heptane mobile phase to investigate the other separation parameters because it was less expensive. In order to achieve sufficient resolution with the best peak shape and shortest retention time, mixtures of the organic modifiers, methanol and ethanol, in heptane were examined. Fig. 2 showed the trends of changing the ratio of the alcohol mixture on the resolution and retention of the separation. A mixture of ethanol and methanol at 2:1 ratio provided excellent results using heptane at a column temperature of 25 °C.

Column temperature often plays an important role for efficiency and selectivity in chiral separation. The trends of increasing temperature on the efficiency and retention were evidenced by changing the column temperature from 25 °C to 40 °C. As shown in Fig. 3, increasing column temperature both sharpened the peaks and reduced the retention times.

After optimization of the resolution using column temperature and mixing of alcohols in heptane, we replaced heptane with ethoxynonafluorobutane (ENFB) to make the method suitable for LC–APCI–MS–MS analysis. Fig. 4 showed that the stereoselectivity was mostly unchanged with the substitution which was consistent with Kagan and Armstrong's findings [23]. However, the retention factors were significantly larger in the ENFB mobile phase at the constant organic modifier composition, which was different from Armstrong's observation for different enantiomers on Chirobiotic V column. The total alcohol content was increased accordingly. In addition, the optimal column temperature and ratio of the alcohols were interrelated and varied slightly with the use of ENFB. The final NP-HPLC–MS–MS method that not only resolved the stereoisomers, but also the potential metabolite, used 63% 3:1 of ethanol:methanol and 37% ENFB at the column temperature of 50 °C. The NP-HPLC–APCI–MS–MS chromatogram is shown in Fig. 5.

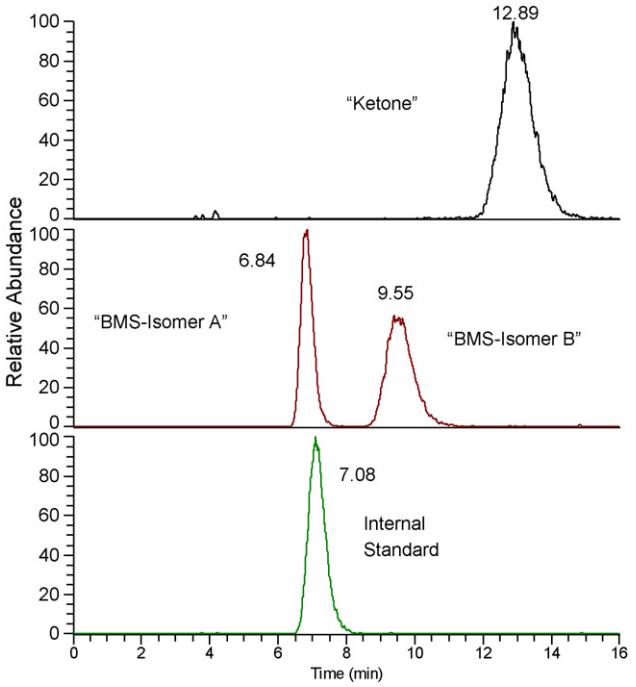

Fig. 5. NP-LC/MS–MS chromatograms of the ketone metabolite (top), BMS-Isomer-A and BMS-Isomer-B (middle) and the internal standard (bottom). Mobile phase: 63% of 3:1 mixture of ethanol and methanol with 37% ENFB at the column temperature of 60 °C.

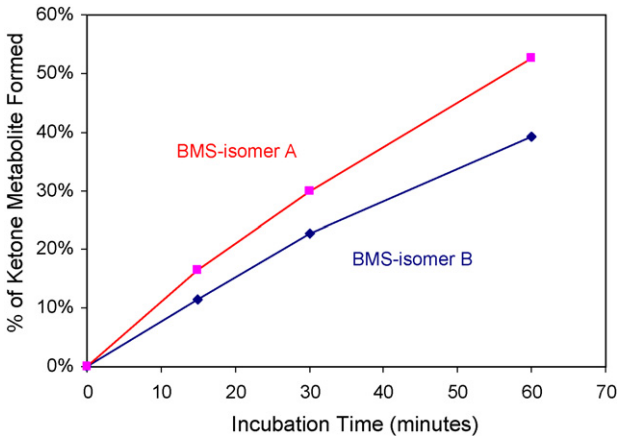

Fig. 6. Rate of the ketone metabolite formation from "BMS-Isomer-A" and "BMS-Isomer-B".

3.2. NP-LC–MS–MS method qualification

Linear dynamic ranges of 5–1000 ng/mL for "BMS-Isomer-A" and the ketone metabolite, and 10–1000 ng/mL for "BMS-Isomer-B" were demonstrated. The response ratios (peak area of the analyte over the internal standard) of the standards were fitted to a linear regression weighted by reciprocal concentration ($1/x$) to generate a standard calibration curve. The correlation coefficient (R^2) was equal to or greater than 0.997. The lower limit of quantitation (LLOQ), defined as the concentration at signal to noise ratio (S/N) of 10:1, was measured to be equivalent to the lowest point of the standard curve, 5 ng/mL for "BMS-Isomer-A" and the ketone, and 10 ng/mL for "BMS-Isomer-B".

The results for the precision and accuracy of the analysis are shown in Table 1. The precision in terms of relative standard deviation (R.S.D.) was <5% at and above the nominal concentration of 80 ng/mL, and <20% at 8 ng/mL. The mean bias was within 15% for both stereoisomers and their metabolite.

Acceptable matrix interferences were demonstrated using "BMS-Isomer-A" and the ketone as the ratio of responses (peak area ratio of the analytes over the internal standard) with and without the matrix were 0.87 and 0.76, respectively. Less than 25% ion suppression or interference was acceptable for the assay. Extraction recoveries were found to be 75% for "BMS-Isomer-A" and 81% for the ketone metabolite, which were also acceptable.

Finally, the stability was checked to ensure the stability of the analytes during the analysis under the sample processing conditions. Acceptable recovery demonstrated the stability of the analytes during the sample preparation. Furthermore, no significant changes in peak retention time and peak area ratio were observed during the 24-h run covering the entire analysis time.

Although only partial validation was performed due to the time and sample quantity constraint, the method was qualified for the purpose of the study, and consequently implemented for the exploratory *in vitro* intrinsic metabolic clearance study.

3.3. In vitro intrinsic metabolic clearance study

An *in vitro* study was performed for each stereoisomer using the NP-HPLC–MS–MS method to examine any stereospecific intrinsic metabolic clearance as well as any chiral inversion. No conversion at the chiral center was observed. However, both molecules

metabolized and formed the ketone metabolite. Furthermore, as shown in Fig. 6, each was found to metabolize at a different rate.

4. Conclusion

Normal-phase chromatography using polysaccharide-type CSPs has been the most successful mode of separation for drug stereoisomers in the pharmaceutical industry. Reversed-phase HPLC–MS–MS has been the primary quantitative technique for bioanalysis due to its great sensitivity and specificity. However, the incompatibility between the traditional normal-phase solvent, hexane or heptane, and mass spectrometry has prevented the potentially powerful combination of normal-phase HPLC with MS–MS detection for stereoselective bioanalysis. We demonstrated that substituting ethoxynonafluorobutane (ENFB) for hexane or heptane can be a viable solution to the problem. The chemical characteristics of ENFB, such as no flashpoint and low flammability, make it ideally suited for APCI–MS detection. ENFB provided comparable stereoselectivity, however, yielded significantly longer retention than heptane at the same percent of alcohol modifiers in our study. A comparable enantioselective resolution using ENFB-substituted mobile phase can be easily obtained by modification of a heptane-based method. The ability to easily adapt a heptane-based method to ENFB permits method screening and development with the less expensive solvent. In this study, we developed and qualified a stereoselective NP-HPLC–MS–MS method using ENFB to quantitatively assess the metabolic stability of a pair of Bristol Myers Squibb proprietary drug stereoisomers, which demonstrated for the first time the practical application and usefulness of ENFB for stereoselective bioanalysis in drug discovery. Combining the success of normal-phase chiral separation with the power of HPLC–MS–MS provides an effective and valuable technique for stereoselective bioanalysis.

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