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Short Communication

Ion Mobility Spectrometry–Mass Spectrometry Analysis for the Site of Aromatic Hydroxylation

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ABSTRACT

Hydroxylated metabolites often retain the pharmacological activity of parent compound, and the position of hydroxylation determines the formation of chemically reactive intermediates, such as quinones and analogs, from para- and/or ortho-hydroxylation of phenols or arylamines. Therefore, the identification of exact position of hydroxylation is often required at the early development stage of new drug candidates. In many cases, liquid chromatography–tandem mass spectrometry (LC-MS/MS) provides identical MS/MS spectra among isomeric hydroxylated metabolites, and therefore, it alone cannot unequivocally identify the exact position(s) of hydroxylation. Ion mobility spectrometry (IMS), integrated with LC-MS/MS, recently showed the capability of separating isomeric species based on differences in their drift times from IMS, which are linearly proportional to the collision cross-section (CCS) reflecting physical size and shape. In the present study, a chemical derivatization of isomeric hydroxylated metabolites with 2-fluoro-*N*-methyl pyridinium *p*-toluenesulfonate was found to confer distinct theoretical CCS value on each isomer by forming corresponding *N*-methyl pyridine (NMP) derivative. The regression lines established by the comparison between theoretical CCS values and observed drift times from IMS for each set of parent compound (labetalol, ezetimibe, atorvastatin, and warfarin) and its MS/MS product ions accurately and selectively projected the actual drift times of NMP derivatives of corresponding aromatic or isomeric hydroxylated metabolites. The established method was used for the accurate assignment of predominant formation of 2-hydroxylated metabolite from imipramine in NADPH- fortified human liver microsomes. The present application expands the versatility of LC-IMS-MS technique to the structure identification of isomeric hydroxylated metabolites at the early stage for drug development.

Introduction

The fragment analysis using liquid chromatography–tandem mass spectrometry (LC-MS/MS) is an effective way of metabolite identification, especially for the rapid identification of metabolites during the early period of drug discovery and development (for review of fragment analyses, Prakash et al., 2007). Although the tandem mass spectrometry (MS/MS) analysis provides structure information on the metabolites by comparing the increase or decrease of the molecular weight of afforded product ions, it does not always provide unambiguous information on the unequivocal characterization for the isomeric substituted molecules, such as hydroxylated metabolites, which often afford identical MS/MS fragments. The recent meta-analysis for the metabolic reactions of over 1000 different drugs and other xenobiotics revealed that aromatic hydroxylation plays a predominant role in generating pharmacologically active metabolite(s) (~30% of all active metabolites), suggesting that the hydroxylated metabolite often retains the target affinity and pharmacological activity of the parent compound (Testa et al., 2012). In addition, the same analysis indicated that the formation of quinones or analogs that is generated from para- and/or ortho-hydroxylation of phenols or arylamines accounts for over 40% of all toxic and/or reactive metabolites. Therefore, the identification of the exact position of hydroxylation is often required at discovery stage from efficacy and safety points of view.

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Recently, we developed a novel method to identify the site of isomeric glucuronide conjugation by ion mobility spectrometry (IMS) integrated with LC-MS/MS: the most likely isomer of glucuronide conjugate was identified from the comparison between actual IMS drift time of target conjugate and the theoretical value of collision cross-section (CCS) calculated by MOBCAL on the generated regression line from the relationship between drift times and CCS values for the set of parent compound and its MS/MS fragments (Shimizu et al., 2012). The regression line–based approach eliminated potential variation in the calculation of three-dimensional (3D) structure under vacuum phase among adopted 2D-3D conversion methods and inherent error ratios in the theoretical CCS values by the modeling, both precluding IMS technology from the routine analysis without the calibration by authentic standards at the early development stage. The theoretical drift time from IMS is linearly proportional to the value of CCS when the masses of ions are much larger than the buffer gas under the same IMS condition, and the value of CCS depends on the physical shape and size of 3D structure of analyte (Smith et al., 2009). During the course of application of IMS analysis to the identification of isomeric hydroxylated metabolites, it was found that the theoretical values of CCS had little difference among isomers, likely because of the subtle difference in the physical shape and/or size of 3D structures among hydroxyl isomers, compared with that among isomers of glucuronide conjugate. Consistently, the isomer separation of hydroxylated metabolites of ondansetron required

ABBREVIATIONS: CCS, collision cross section; FMPTS, 2-fluoro-1-methyl pyridinium *p*-toluenesulfonate; IMS, ion mobility spectrometry; IMS-MS, ion mobility spectrometry–mass spectrometry; LC-IMS-MS, liquid chromatography–ion mobility spectrometry–mass spectrometry; LC-MS, liquid chromatography–mass spectrometry; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MS/MS, tandem mass spectrometry; *m*/z, mass-to-charge ratio; NMP, *N*-methylpyridyl; TM, trajectory method; UPLC, ultraperformance liquid chromatography.

calibration with authentic standard for each isomer to improve precision of determined drift times by ion mobility spectrometry–mass spectrometry (IMS-MS) method (Dear et al., 2010).

Chemical derivatization in combination with LC-MS/MS has been well established for the enhancement of detection sensitivity by introducing an easily ionizable function or a constantly charged group into the intact analyte (Lampinen-Salomonsson et al., 2006). Lin et al. (2007) used 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FMPTS) to chemically derivatize phenolic group of estrogens and improved sensitivity by LC-MS for 17β-estradiol, estrone, and 17α-ethinylestradiol. FMPTS reacts with aromatic hydroxyl group, forming *N*-methylpyridyl (NMP) ether ion of *m/z* (mass-to-charge ratio) $[M+92]^+$ by nucleophilic substitution (Quirke et al., 1994; Lin et al., 2007). NMP moiety possesses similar size to glucuronic acid by 3D modeling, and it also enhances the sensitivity of analyte by LC-MS because of the presence of cationic quarternary amine. Therefore, the chemical derivatization with FMPTS likely confers distinct physical shape and CCS value on each isomer of hydroxylated metabolites as seen in the isomeric glucuronide conjugates, which enables regression-based liquid chromatography–ion mobility spectrometry–mass spectrometry (LC-IMS-MS) analysis to identify the exact position of hydroxylation on the aromatic moiety without authentic standards.

In this communication, we present an approach for the IMS-MS technology to the identification of exact site(s) of aromatic hydroxylation: the chemical derivatization with FMPTS was found to selectively derivatize aromatic hydroxyl group by forming NMP ether ion of *m/z* $[M+92]^+$, and the drift times of derivatized isomeric hydroxylated metabolites determined by IMS were selectively predicted from regression-based LC-IMS-MS analysis (Shimizu et al., 2012) based on the corresponding theoretical CCS values. The present method, therefore, expands the versatility of LC-IMS-MS technique to the structure identification of isomeric hydroxylated metabolites, which is often required during the early stage for new drug development as pharmacologically and/or toxicologically important molecules.

Materials and Methods

Materials. Atorvastatin, ortho-hydroxy atorvastatin calcium salt, and para-hydroxy atorvastatin calcium salt were purchased from TLC PharmaChem Inc. (Vaughan, ON, Canada). Labetalol hydrochloride, imipramine hydrochloride, and warfarin were purchased from Sigma-Aldrich (St. Louis, MO). Authentic metabolites of 6-hydroxy warfarin, 7-hydroxy warfarin, and 8-hydroxy warfarin were purchased from Salford Ultrafine Chemicals & Research Ltd. (Manchester, UK). Ezetimibe and 2-hydroxy-imipramine were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). FMPTS (2-Fluoro-1-methylpyridinium *p*-toluenesulfonate) was purchased from Tokyo Chemical Industries CO., LTD. (Tokyo, Japan). Triethylamine was purchased from Nacalai Tesque (Kyoto, Japan). Glucose-6-phosphate dehydrogenase, $NADP^+$, and D-glucose-6-phosphate disodium salt were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Pooled human liver microsomes from 50 individual donors (Lot No.0910398; 31 males and 19 females) were purchased from XenoTech LLC (Lenexa, KS). All other reagents were of analytical grade.

Analytical Procedure. The LC-IMS-MS was conducted on an Acquity ultraperformance liquid chromatography (UPLC) system equipped with a binary pump, autosampler, thermostat, and column compartment (Waters Corp., Milford, MA), coupled with Synapt G2 high definition mass spectrometer (Waters Corp., Manchester, UK) including ion mobility spectrometer and time-of-flight–type high-resolution mass spectrometry. Solvent A was 0.1% formic acid, and solvent B was 0.1% formic acid in acetonitrile. Chromatographic separations were performed on an Acquity UPLC BEH C18 (1.7 μm, 2.1×50 mm; Waters Corp., Dublin, Ireland) using a 10-minute gradient at a flow rate of 0.4 ml/min, starting at 10% solvent B, linearly increasing to 70% solvent B over 7 minutes, followed by 90% solvent B for 1.5 minutes, and re-equilibrated for 1.5 minutes. Eluents from UPLC during pre- and postanalysis (0.5 and 7 minutes, respectively) were discarded through the diversion valve. IMS analyses were performed on a Synapt G2 high definition mass spectrometer under the conditions described as follows: capillary voltage, 3 kV; cone voltage, 30 V; trap collision energy, 0 V or 10-40 V ramping; transfer collision energy, 0 V; trap/transfer gas, argon; IMS gas, nitrogen; IMS T-wave speed, 900 m/s; IMS T-wave height, 40 V; IMS gas flow, 20 or 35 mL/min; IMS-MS acquisition time, 7 minutes after samples injected onto column.

Calculations of CCS. Theoretical values of CCS were calculated using the method suggested by Dear et al. (2010). In brief, the conformation for each molecule was energy minimized with the MMFF94 force field, followed by the extraction of 3D coordinate sets of each atom. Theoretical CCS values were then calculated by the open source software MOBCAL (Jarrold Group, Indiana University - Bloomington) (Mesleh et al., 1996) with 3D coordinate data sets. The MOBCAL could be downloaded from http://www.indiana.edu/~nano/software.html freely and operates on Microsoft Windows PC. The MOBCAL output is based on three different models/algorithms, including projection approximation, exact hard sphere scattering, and the trajectory method (TM). Among three outputs, the MOBCAL TM has been demonstrated to provide most accurate projections of CCS values with percentage differences of $<4.5\%$ for isomeric organoruthenium anticancer complexes (Williams et al., 2009) and of $<1\%$ for ondansetron and its hydroxylated metabolites (Dear et al., 2010). Therefore, the outputs of MOBCAL TM were used for the projections in this study.

Regression Line for CCS and IMS Drift Time. Although the TM is generally accepted as the most accurate method of calculating the theoretical CCS values among multiple MOBCAL outputs, the relationship between CCS and IMS drift times cannot serve as an absolute basis for the exact identification of unknown structure of target analyte. Therefore, in addition to the TM-based CCS value for the parent compound, those values for its MS/MS fragments were plotted against corresponding actual IMS drift times to generate the regression line. Thus, generated regression line facilitates accurate projection of IMS drift time of unknown analyte (NMP derivative of hydroxylated metabolite) by the comparison with (multiple) theoretical CCS value(s) of potential structure(s).

Derivatization with FMPTS. The compound solution in acetonitrile (1000 μl, 10 μM) was added to 1 μl of FMPTS solution (10 mM in dimethyl sulfoxide, 10 equivalents) and 1 μl of triethylamine. The resultant mixture was shaken for 5 minutes at room temperature. The reaction mixture was then injected onto the LC-IMS-MS system for analysis.

Incubation of Imipramine with Human Liver Microsomes. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 3 mM magnesium chloride, 1.0 mg/ml human liver microsomal protein, and NADPH-generating system (1 mM $NADP^+$, 1 unit glucose-6-phosphate dehydrogenase, and 10 mM D-glucose-6-phosphate disodium salt). The reaction was initiated by the addition of imipramine hydrochloride (1 mM in dimethyl sulfoxide) to achieve 10 μM at final concentration. The reaction was conducted at 37°C for 60 minutes and terminated by the addition of two volumes of acetonitrile/methanol (2:1, v/v). The supernatant was separated by centrifugation (13,800*g*, 10 minutes, 4°C), followed by evaporation under the stream of nitrogen gas. The residue was dissolved in acetonitrile and dried over magnesium sulfate, followed by centrifugation to remove powder of magnesium sulfate. The resultant supernatant was treated with FMPTS for the NMP derivatization as described in the preceding section.

Results and Discussion

A novel approach in the present study to the identification of the site of isomeric hydroxylation is to combine phenolic hydroxyl group selective derivatization by the simple incubation with a chemical derivatization reagent, FMPTS, NMP ether derivatives and the IMS analysis. The derivatization conferred distinct theoretical CCS value calculated by MOBCAL on each isomeric hydroxylated metabolite, otherwise not amenable to the discrimination because of subtle difference in the CCS values among isomers. The regression-line based projection method, which had been established by the comparison between theoretical CCS values and observed drift times in IMS for multiple sets of glucuronide conjugate and its MS/MS fragments (Shimizu et al., 2012), accurately and selectively identified the NMP

derivatives of phenolic metabolites of labetalol and ezetimibe and isomeric hydroxylated metabolites of atorvastatin, warfarin, and imipramine.

FMPTS is known to react with the hydroxyl group (Mukaiyama et al., 1975), resulting in the formation of NMP ether ion of *m/z* $[M+92]^+$ by nucleophilic substitution (Quirke et al., 1994; Lin et al., 2007). FMPTS reacts with primary/secondary alcohols (forming NMP ether derivatives) and primary/secondary/tertiary thiols (forming NMP sulfide) (Quirke et al., 1994). In addition, the sensitivity for estrogens, such as 17β-estradiol, estrone, and 17α-ethinylestradiol, by LC-MS was improved by the chemical derivatization technique with FMPTS to modify the phenolic group of estrogens so that their ionization efficiency was increased (Lin et al., 2007). Under the simple condition in the present study that the analyte and FMPTS were incubated in triethylamine/acetonitrile for 5 minutes at room temperature, the derivatization selectively afforded NMP derivatives with phenol groups of labetalol and ezetimibe (Fig. 1), both possessing phenolic and aliphatic alcohols for the potential sites of nucleophilic substitution by FMPTS. On the other hand, either imipramine or warfarin did not react with FMPTS under the same condition. To ensure the accurate projection of thus formed NMP derivatives of labetalol and ezetimibe based on the regression line-based LC-IMS-MS analysis, the linearity of regression line between CCS values and IMS drift times was confirmed for both sets of parent compound, its MS/MS fragments, and NMP derivative of phenolic hydroxyl group of parent compound [labetalol (Fig. 1B) and ezetimibe (Fig. 1C)]. The drift times of labetalol 2 (Fig. 1B) and its six MS/MS fragments 3–8 (drift time, 7.07, 6.91, 6.75, 5.67, 5.45, 4.86, and 4.70 minutes, respectively; CCS value, 128.60, 124.99, 122.15, 88.350, 84.645, 71.466, and 66.066 Å^2, respectively) accurately projected actual drift time of NMP derivative of labetalol 1 (8.15 minutes) at the theoretical CCS value (155.90 Å^2) with predicted drift time of 8.07 minutes from regression line (drift time = 0.0372 CCS + 2.2715; R^2 = 0.9960). Similarly, ezetimibe (10 in Fig. 1C) and its MS/MS fragments, 11, 12, and 13 (drift time, 7.88, 7.61, 6.16, and 4.43 minutes, respectively; CCS value, 144.09, 140.89, 107.49, and 63.648 Å^2, respectively) projected actual drift time of NMP derivative of ezetimibe 9 (9.13 minutes) at the theoretical CCS value (172.31 Å^2) with predicted drift time of 8.99 minutes based on the regression line (drift time = 0.0423 CCS + 1.6995; R^2 = 0.9975).

The present chemical derivatization to generate NMP derivatives conferred distinct theoretical CCS value on each isomer of potential hydroxylated metabolites of atorvastatin, warfarin, and imipramine (Table 1), otherwise not amenable to the discrimination by LC-IMS-MS because of the subtle difference in the CCS value among isomers of intact hydroxylated metabolites. Figure 2 shows the relationships and linear regression lines between CSS values and IMS drift times for the sets of parent drug [atorvastatin (Fig. 2A), warfarin (Fig. 2B), and imipramine (Fig. 2C)], its MS/MS fragments, its authentic hydroxylated metabolites (for atorvastatin and warfarin), and the corresponding NMP derivatives. Atorvastatin (Fig. 2A, 18) is known to afford two hydroxylated metabolites by CYP3A (Riedmaier et al., 2011). By fragment analyses, these two hydroxylated metabolites were both assigned as hydroxylations of terminal benzene, but the position of hydroxylation was not determined by LC-IMS-MS analysis. The drift times of atorvastatin (18 in Fig. 2A) and its MS/MS fragments (19–24) were 7.24, 6.59, 6.43, 6.10, 5.72, 5.29, and 4.97 minutes, respectively, and the corresponding CCS values were 182.25, 156.28, 153.06, 148.85, 137.52, 127.33, and 113.34 Å^2, respectively. The relationship generated a linear regression line (drift time = 0.0350 CCS + 0.9527) with high correlation coefficient (R^2 = 0.9802), which allowed for an accurate identification of three potential isomers of NMP derivatives of ortho-, meta-, and para-hydroxylated metabolites with predicted

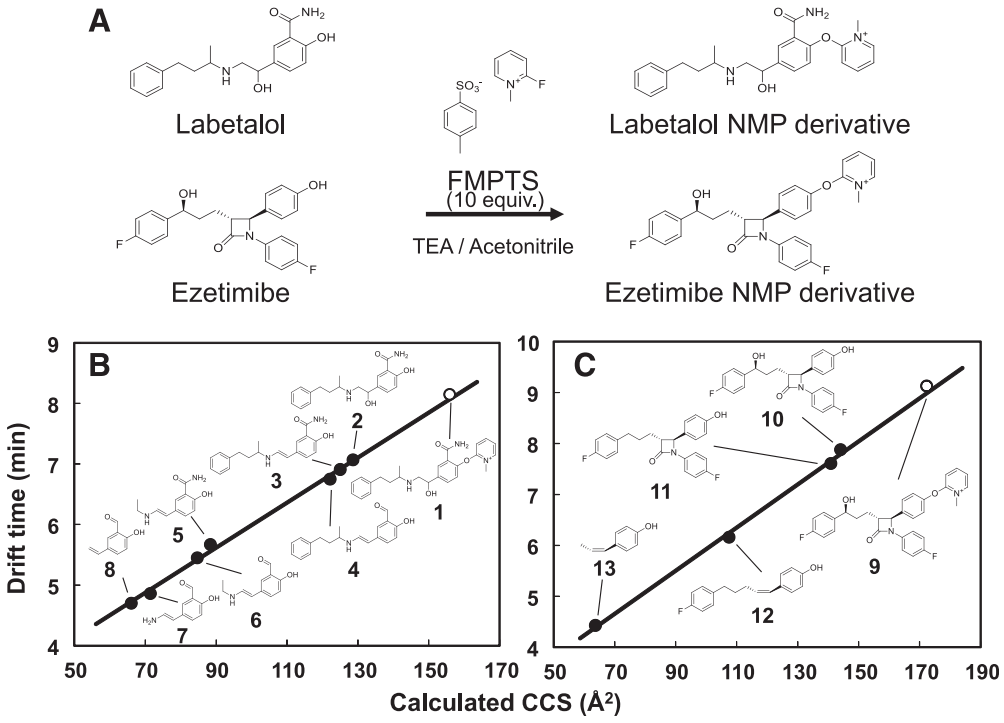

Fig. 1. Scheme of chemical derivatizations of labetalol and ezetimibe with FMPTS, forming corresponding NMP derivatives (A) and plot of the drift times (minutes) against calculated CCS values (Å^2) for the singly charged ions of NMP derivatives of hydroxylated metabolites, intact hydroxylated metabolites, parent ion, and its MS/MS product ions of labetalol (B) and ezetimibe (C). Linear regression for labetalol 2 and its MS/MS fragments (3–8) gave drift time = 0.0372 CCS + 2.2715 (R^2 = 0.9960). NMP derivative at phenolic alcohol (1) is shown by open circle (B). Linear regression for ezetimibe (10) and its MS/MS fragments (11–13) gave drift time = 0.0423 CCS + 1.6995 (R^2 = 0.9975). NMP derivative at phenolic alcohol (9) is shown by open circle (C).

TABLE 1

TM-based theoretical CCS values for the potential hydroxylated metabolites of parent drugs (atorvastatin, warfarin, and imipramine) and their corresponding *N*-methyl pyridine derivatives

Parent Compound	Potential Metabolites	TM-Based Calculated CCS	
		Intact Metabolites	*N*-Methyl Pyridine Derivatives
		$Å^2$	
Atorvastatin	Ortho-hydroxy atorvastatin	182.29	200.26
	Meta-hydroxy atorvastatin	188.36	206.61
	Para-hydroxy atorvastatin	187.73	214.55
Warfarin	6-Hydroxy warfarin	110.42	140.62
	7-Hydroxy warfarin	110.23	142.60
	8-Hydroxy warfarin	109.84	135.92
Imipramine	1-Hydroxy imipramine	113.37	138.85
	2-Hydroxy imipramine	114.09	144.42
	3-Hydroxy imipramine	113.38	133.48
	4-Hydroxy imipramine	111.86	134.87

drift times of 7.96, 8.18, and 8.46 minutes, respectively. The regression line thus generated accurately projected actual drift times of two NMP derivatives of authentic para- (Fig. 2A, 14) and ortho- (Fig. 2A, 15) hydroxylated metabolites of atorvastatin at 8.59 and 7.99 minutes, respectively. Warfarin 31 is known to afford many hydroxylated metabolites, including 4′-, 6-, 7-, 8-, and 10-hydroxy warfarin (Daly and King, 2003). Both 4′-hydroxy and 10-hydroxy warfarin were distinguished from other three hydroxylated metabolites (6-, 7-, and 8-hydroxy warfarin) by LC-MS/MS, whereas the positions of hydroxylation of 6-, 7-, 8-hydroxy warfarin could not be assigned by LC-IMS-MS (Table 1; 28, 29, and 30 in Fig. 2B). The drift times of warfarin (31 in Fig. 2B) and its MS/MS fragments (32, 33, and 34) were 6.75, 6.05, 4.81, and 4.86 minutes, respectively, and the corresponding CCS values were 106.5, 92.886, 64.436, and 71.559 $Å^2$, respectively. The relationship generated a linear regression line (drift time = 0.0486 CCS + 1.5437) with high correlation coefficient (R^2 = 0.9832), which allowed for an accurate identification of three potential isomers of NMP derivatives of 6-, 7-, and 8-hydroxylated

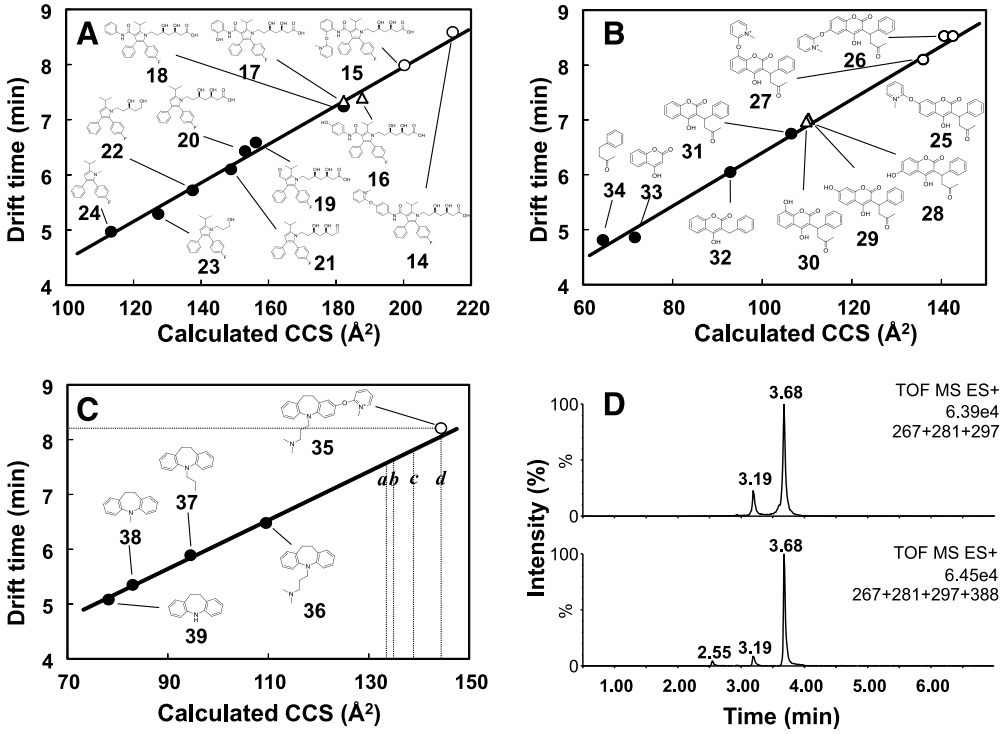

Fig. 2. Plot of the drift times (minutes) against calculated CCS values ($Å^2$) for the singly charged ions of NMP derivatives of hydroxylated metabolites, intact hydroxylated metabolites, parent ion, and its MS/MS product ions of atorvastatin (A), warfarin (B) and imipramine (C). LC-MS chromatograms of the incubated sample of imipramine with NADPH-fortified human liver microsomes before (upper panel) and after (lower panel) chemical derivatization of metabolically formed hydroxylated metabolites by FMPTS (D). Linear regression for atorvastatin (18) and its MS/MS fragments (19–24) gave drift time = 0.0350 CCS + 0.9527 (R^2 = 0.9802). Two NMP derivatives of ortho- and para-hydroxy atorvastatin (14 and 15) are shown by open circles, and two intact hydroxylated metabolites (16 and 17) are shown by open triangles (A). Linear regression for warfarin (31) and its MS/MS fragments (32, 33, and 34) gave drift time = 0.0486 CCS + 1.5437 (R^2 = 0.9832). Three NMP derivatives of hydroxylated metabolites (25, 26, and 27) are shown by open circles, and three intact hydroxylated metabolites (28, 29, and 30) are shown by open triangles (B). Linear regression for imipramine (36) and its MS/MS fragments (37, 38, and 39) gave drift time = 0.0444 CCS + 1.6503 (R^2 = 0.9957). Theoretical CCS values for 3-, 4-, 1-, and 2-hydroxylated imipramine are plotted by dotted lines at 133.48 (*a*), 134.87 (*b*), 138.85 (*c*), and 144.42 (*d*) $Å^2$, respectively (Table 1). The NMP derivative of metabolically formed hydroxylated metabolite agreed well with that predicted for 2-hydroxyimipramine (35), shown by open circle (C).

metabolites with predicted drift times of 8.38, 8.47, and 8.15 minutes, respectively. The actual drift time of three NMP derivatives of authentic 6-, 7-, and 8-hydroxylated metabolite were 8.53, 8.53, and 8.10 minutes, respectively, indicating that the regression line projected the drift time for 8-hydroxy warfarin separately from 6- or 7-hydroxylated metabolite.

The regression line–based approach was applied to the identification of positions of hydroxylated metabolites formed from imipramine in NADPH-fortified human liver microsomes. Figure 2D shows representative LC-MS chromatograms of the incubation sample before and after NMP derivatization. The peak at retention time of 3.68 minutes on both panels gave molecular ions $[M+H]^+$ at *m/z* 281 and 267, which corresponded to the intact imipramine and *N*-desmethyl imipramine, respectively. The secondary peak at 3.19 minutes gave molecular ions $[M+H]^+$ at *m/z* 297, which corresponded to the intact hydroxylated metabolite(s) of imiparamine on benzene ring by LC-MS analyses, whereas the structural information by MS/MS did not afford unequivocal identification of exact position of hydroxylation among four potential isomers of hydroxylated imipramine (i.e., 1-, 2-, 3-, and 4-position in Table 1). The peak at 2.55 minutes on the lower panel in Fig. 2D afforded molecular ion $[M+H]^+$ at *m/z* 388, corresponding to the addition of *N*-methylpyridyl ether ion of *m/z* $[M+92]^+$ to metabolically formed hydroxylated metabolite of imipramine in NADPH-fortified human liver microsomes. As shown in Fig. 2C, imipramine 36 and its MS/MS fragments (37, 38, and 39) generated a regression line (drift time = 0.0444 CCS + 1.6503; $R^2 = 0.9957$) based on the relationship between drift times (6.48, 5.89, 5.35, and 5.08 minutes, respectively) and CCS values (109.51, 94.507, 82.984, and 78.197 $Å^2$, respectively). The theoretical CCS values of 1-, 2-, 3-, and 4-hydroxylated imipramine calculated by MOBCAL were 138.85 (Fig. 2C), 144.42, 133.48, and 134.87 $Å^2$, respectively, and predicted IMS drift times were 7.82, 8.06, 7.58, and 7.63 munutes, respectively, by the regression line. The actual drift time of the peak for the NMP derivative at 2.55 minutes (Fig. 2D) was 8.21 minutes, suggesting that the major hydroxylated metabolite of imipramine in the incubated sample with human liver microsomes was the 2-hydroxy imipramine 35, which is consistent with both in vitro and clinical data that 2-hydroxylated imipramine (and its glucuronide conjugate in vivo) is a predominant hydroxylated metabolite observed in human liver microsomes (Koyama et al., 1997), plasma, and urine (Sutfin et al., 1984). Further analyses with authentic 2-hydroxyimipramine confirmed the regression line–based metabolite identification by the actual drift time of 8.21 minutes, in excellent agreement with the corresponding calculated value.

In conclusion, the chemical derivatization forming *N*-methylpyridyl ether of aromatic hydroxyl groups conferred distinct theoretical CCS value on each potential isomer of hydroxylated metabolites, which enabled unequivocal identification of the analyte of interest among isomeric hydroxylated metabolites by drift times in IMS based on the previously established regression-based LC-IMS-MS method (Shimizu et al., 2012). The present method offers an extra degree of analytical opportunity for the isomeric aromatic hydroxylated metabolites by one-step simple derivatization for LC-IMS-MS analysis and has proven to be a powerful tool for preliminary elucidation of the hydroxylated position in the biologic matrices, especially during the early development stage, without synthesis of authentic metabolites or isolation of metabolites for NMR spectroscopy. Further studies are warranted to evaluate potential and versatility of chemical derivatization in the structural identification of isomeric metabolites by LC-IMS-MS analysis.

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Authorship Contributions

Participated in research design: Shimizu, Chiba.
Conducted experiments: Shimizu.
Contributed new reagents or analytic tools: Shimizu.
Performed data analysis: Shimizu.
Wrote or contributed to the writing of the manuscript: Shimizu, Chiba.

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