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Comparative metabolism of sildenafil in liver microsomes of different species by using LC/MS-based multivariate analysis

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ABSTRACT

Sildenafil metabolism in liver microsomes obtained from different species was studied *in vitro* and compared using liquid chromatography–mass spectrometry (LC/MS) and multivariate statistical analysis. Sildenafil (1, 5, and 25 μM) was incubated with rat, mouse, dog, monkey, and human liver microsomes along with NADPH, and the reaction mixtures were analyzed by LC/MS to obtain species-specific metabolic profiles of sildenafil. A total of 12 metabolites were detected and their peak area ratio values were used as variables for multivariate analyses to evaluate the interspecies differences in sildenafil metabolism. Principal components analysis of the metabolic profiles showed that the mouse samples were generally clustered closer to the human samples on the principal component score plot. Similarity index (SI) indicated that sildenafil metabolism in mice, compared to the other animals, was highly analogous (SI = 0.764 at 25 μM) to that in humans. These results suggest that LC/MS-based multivariate analytical approaches are useful for the evaluation of interspecies differences in the metabolism of xenobiotics.

1. Introduction

Drug metabolism and pharmacokinetic properties of new chemical entities are extensively investigated at various stages of drug discovery and development [1]. In particular, drug metabolism is important in that it reveals the pharmacological activity and toxicological implications of a drug to ensure that it can be used safely in humans. In the lead discovery and optimization phases, drug metabolism properties are screened using *in vitro* systems such as microsomes, S9 fractions, and/or hepatocytes, and compounds with favorable metabolic stability are selected [2–5]. The *in vitro* systems are used to characterize and compare metabolic pathways across species to determine whether major metabolites in humans are appropriately reflected in nonclinical species selected for safety or toxicological studies [6]. Based on *in vitro* metabolism profiles, *in vivo* metabolism studies are further conducted through the late preclinical and phase I/II clinical trials [1].

Liquid chromatography–mass spectrometry (LC/MS) provides a powerful solution for investigating the metabolism of xenobiotics and is commonly used in various phases of drug discovery and development. Recently, LC/MS analysis has been combined with multivariate chemometric techniques such as principal component analysis (PCA) and used for metabolomic and metabonomic studies [7–11]. These approaches can be used to visualize the enormous datasets collected from biological samples by describing an *n*-dimensional space of the variable metabolic profile as a smaller number of dimensions. Additionally, by using a mathematical technique such as a similarity analysis, the similarity between multi-dimensional datasets can be represented by a score, and the datasets be clearly compared with each other [12,13]. In this context, LC/MS analysis coupled with multivariate techniques can be successfully employed for investigation of xenobiotic metabolism, particularly, comparative metabolism of xenobiotics with numerous metabolites in different species, in which case multiple datasets should be analyzed and compared simultaneously.

Sildenafil is a cGMP-specific phosphodiesterase type 5 (PDE5) inhibitor and well known as a drug used to treat erectile dysfunction [14]. The *in vitro* and *in vivo* metabolism of sildenafil has already been extensively investigated in various species [15,16]. According to a previous study [15], 10 metabolites were detected in experimental animal and human samples, and the major metabolic pathways of sildenafil were piperazine *N*-demethylation, pyrazole *N*-demethylation, loss of a 2-carbon fragment from the piperazine ring (*N*,*N*′-deethylation), oxidation of the piperazine ring, and aliphatic hydroxylation. The metabolic profile of sildenafil metabolism differs among species. Therefore, a multivariate

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analysis approach could help to comprehensively understand and clearly compare sildenafil metabolism in different species.

In the present study, a novel application of PCA and similarity index (SI) was studied to evaluate the interspecies differences in metabolism of a xenobiotic by using sildenafil as a model compound. For a comparative metabolism study, sildenafil was incubated with liver microsomes collected from 5 different species, the reaction mixtures were analyzed by LC/MS, and the metabolic profiling data based on the LC/MS chromatogram were subjected to PCA and similarity analysis.

2. Materials and methods

2.1. Chemicals

Sildenafil citrate (purity, 99.9%) and mirodenafil HCl (purity, >99%) were donated by SK Ltd. (Suwon, Korea). Pooled human, rat, and mouse liver microsomes were obtained from BD Gentest (Woburn, MA, USA), and pooled monkey and dog liver microsomes, from *In Vitro* Technologies, Inc. (Baltimore, MD, USA) (Supplemental Table S1). Glucose-6-phosphate, β-NADP$^+$ and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade and were used as received.

2.2. Biotransformation of sildenafil

Sildenafil (at final concentrations of 1, 5, and 25 μM) was incubated in triplicate with 1 mg/mL microsomal protein in 0.1 M potassium phosphate buffer, pH 7.4, at 37 °C for 90 min in a final incubation volume of 200 μL. The reaction was initiated by the addition of an NADPH-generating system (NGS) containing 0.8 mM β-NADP$^+$, 10 mM glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase to the reaction mixture. After incubation, 5 μL of the internal standard solution (1 μg/mL of mirodenafil) was added, followed by 1 mL of ethylacetate for extraction. The organic layer (900 μL) was collected and evaporated to dryness under nitrogen. The residue was reconstituted with 100 μL of 50% MeOH with 0.1% formic acid and injected into an HPLC column.

2.3. Instruments

The LC/MS system consisted of an HP 1100 series HPLC system (Agilent, Palo Alto, CA, USA) with an LC/MSD ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent, Palo Alto, CA, USA). The column used for the separation was a Hypersil GOLD 3 μM (2.1 mm × 150 mm, Thermo Fisher Scientific Inc, Pittsburgh, PA, USA). The column temperature was maintained at 40 °C by using a thermostatically controlled column oven. The HPLC mobile phases consisted of 0.1% formic acid (A) and 90% acetonitrile in 0.1% formic acid (B), and a gradient program was used for the HPLC separation at a flow rate of 0.22 mL/min. The initial composition of the mobile phases was 20% of B, programmed linearly to 45% of B after 40 min, and held for 1 min. The entire column eluent was directly introduced into an ESI interface through a 50 cm-long PEEK tubing (i.d., 0.13 mm). Nitrogen was used both as the nebulizing gas at 40 psi and as the drying gas with a flow rate of 8 L/min at a temperature of 350 °C. The mass spectrometer was operated in the positive ion mode in a mass range of *m/z* 250–450. Helium was used as collision gas for tandem mass spectrometric experiments. Fragmentation was induced with a resonant excitation amplitude of 0.6 V, followed by an isolation of the precursor ion over a selected mass window of 1 Da.

High-resolution and high-accuracy MS experiments were conducted with Finnigan LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Acquisition and analysis of the data were performed using Xcalibur (Version 2.2, Thermo Fisher Scientific Inc., Waltham, MA, USA). Full-scan MS spectra were acquired for the accurate measurement of the masses of sildenafil and its metabolites. The column, column temperature, HPLC mobile phases, and gradient program were used as described above. Nitrogen at 270 °C was used as sheath and auxiliary gas at 40 and 20 arbitrary units, respectively. The mass spectrometer was operated in the positive ion mode in the mass range of *m/z* 150–450. Ion spray voltage was adjusted to 4500 V. Helium was used as collision gas for the tandem mass spectrometric experiments.

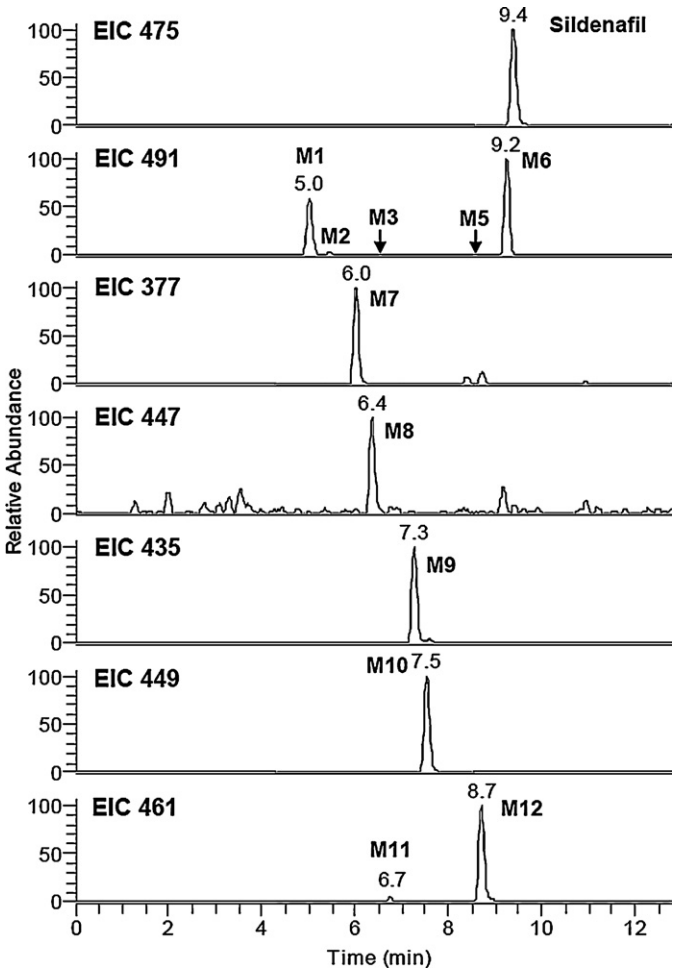

Fig. 1. Representative extracted ion chromatogram of sildenafil and its metabolites produced following the incubation with human liver microsomes.

2.4. Data analysis

For pattern recognition analysis, 14 peaks, including the peaks of sildenafil, the internal standard, and postulated metabolites, were selected on the basis of peak heights (S/N > 5). The peaks were identified by relative retention time and the *m/z* value of protonated ions. The dataset was constructed with the peak-area ratios of the selected peaks. Subsequently, the dataset was evaluated by PCA using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The principal component (PC) score plots of the first 3 PCs were generated from the results of the PCA. The SI between the metabolic profiles of

Table 1
Accurate mass measurement of sildenafil and its metabolites found in human liver microsomes.

Compounds	$[M+H]^+$	ΔM	Measured mass (Da)	Elemental composition	Theo. mass (Da)	Error (ppm)
Sildenafil	475	–	475.21249	$C_{22}H_{31}O_4N_6{}^{32}S_1$	475.21220	0.61
M1	491	+16	491.20726	$C_{22}H_{31}O_5N_6{}^{32}S_1$	491.20712	0.29
M2	491	+16	491.20676	$C_{22}H_{31}O_5N_6{}^{32}S_1$	491.20712	−0.76
M3	491	+16	491.20675	$C_{22}H_{31}O_5N_6{}^{32}S_1$	491.20712	−0.74
M4	491	+16	N.D.	$C_{22}H_{31}O_5N_6{}^{32}S_1$	491.20712	N.D.
M5	491	+16	491.20685	$C_{22}H_{31}O_5N_6{}^{32}S_1$	491.20712	−0.54
M6	491	+16	491.20738	$C_{22}H_{31}O_5N_6{}^{32}S_1$	491.20712	0.54
M7	377	−98	377.12799	$C_{17}H_{21}O_4N_4{}^{32}S_1$	377.12780	0.50
M8	447	−28	447.18083	$C_{20}H_{27}O_4N_6{}^{32}S_1$	447.18090	−0.16
M9	435	−40	435.18098	$C_{19}H_{27}O_4N_6{}^{32}S_1$	435.18090	0.18
M10	449	−26	449.19676	$C_{20}H_{29}O_4N_6{}^{32}S_1$	449.19655	0.47
M11	461	−14	461.19642	$C_{21}H_{29}O_4N_6{}^{32}S_1$	461.19655	−0.28
M12	461	−14	461.19668	$C_{21}H_{29}O_4N_6{}^{32}S_1$	461.19655	0.28

N.D.: not detected in human liver microsomes.

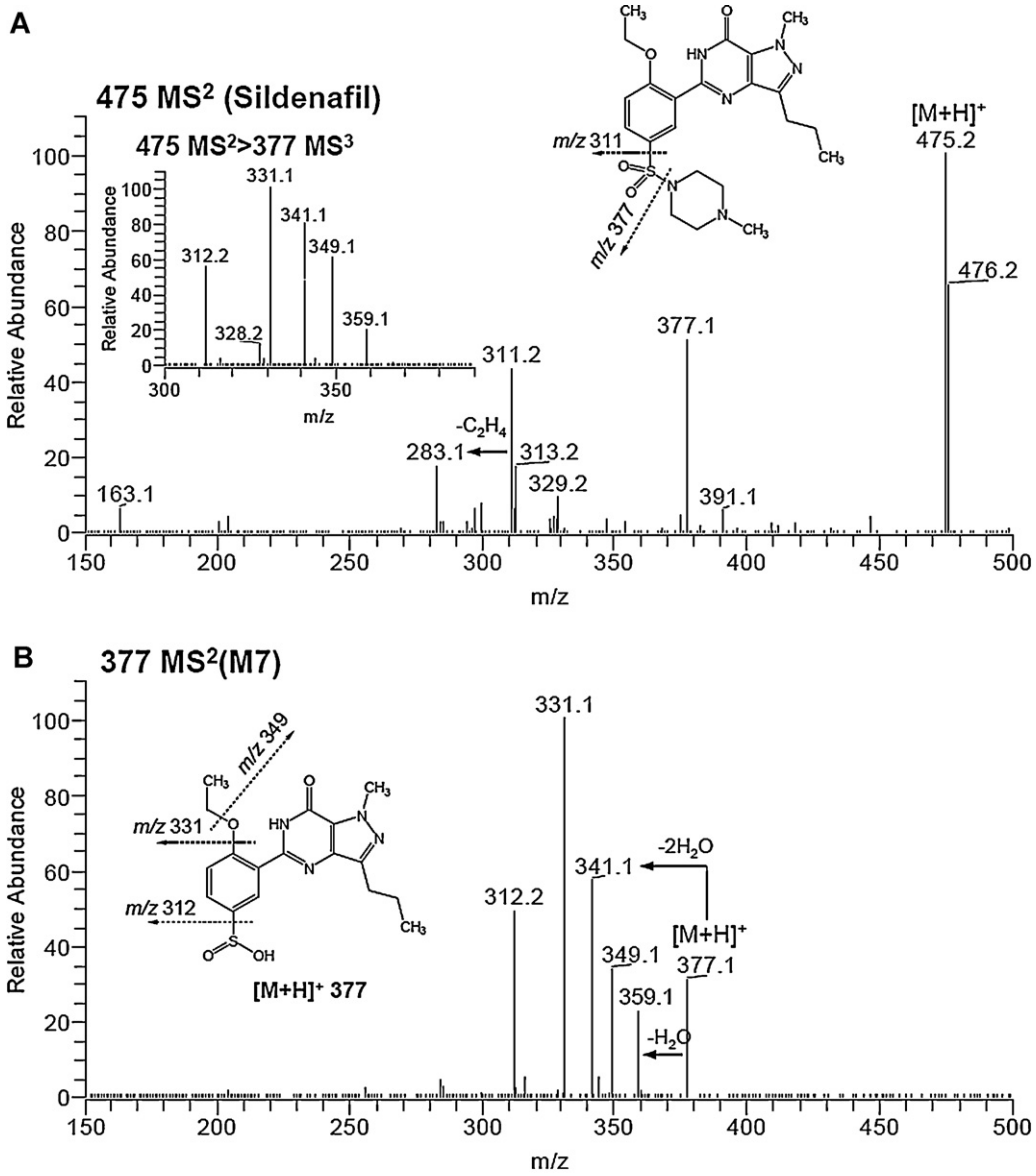

Fig. 2. Collision induced dissociation spectra of protonated sildenafil and M7.

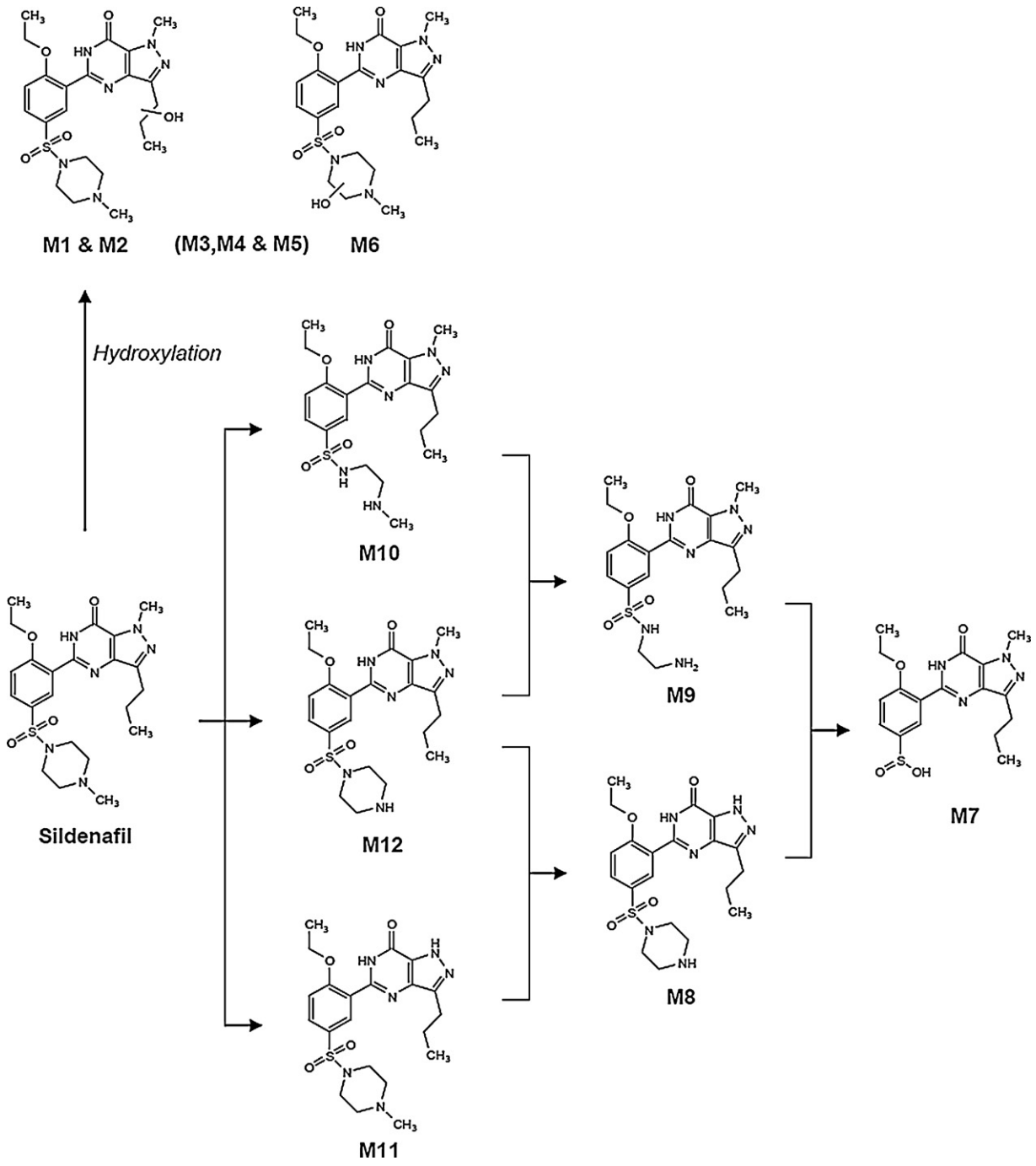

Fig. 3. Postulated metabolic pathway of sildenafil in liver microsomes.

human and the other species was calculated using the Bray–Curtis equation as follows [12]:

$$\mathrm{SI} = 2\frac{\sum_{i=1}^{s}\min(X_{ij}, X_{ik})}{\sum_{i=1}^{s}(X_{ij} + X_{ik})}$$

3. Results

3.1. In vitro metabolic profiling

The incubation of sildenafil with human, mouse, rat, monkey, and dog liver microsomes (HLM, MLM, RLM, MkLM, and DLM) in the presence of NADPH generated 12 metabolite peaks (S/N > 5), shown in Fig. 1. When sildenafil was incubated without microsomes, no metabolites were observed, indicating that all metabolites were generated in the presence of microsomal enzymes (data not shown). In HLM, 11 metabolites, except M4, were found at a concentration of 1–25 μM. In MLM and MkLM, all the metabolites were detected. Eight metabolites, except M1, M3, M4, and M11, in RLM and 7 metabolites, including M6–M12, in DLM were detected (Supplemental Fig. S1).

The MH^+ ions of the metabolites in the HLM sample were identified by high resolution/full-scan MS detection (Table 1). The metabolites M1–M6 were observed at *m/z* 491, indicating monohydroxylation on the aliphatic or piperazine moiety; M10, at *m/z* 449, indicating piperazine *N*,*N*-deethylation; M11 and M12, at *m/z* 461, by piperazine and pyrazole *N*-demethylation; M9, at *m/z* 435,

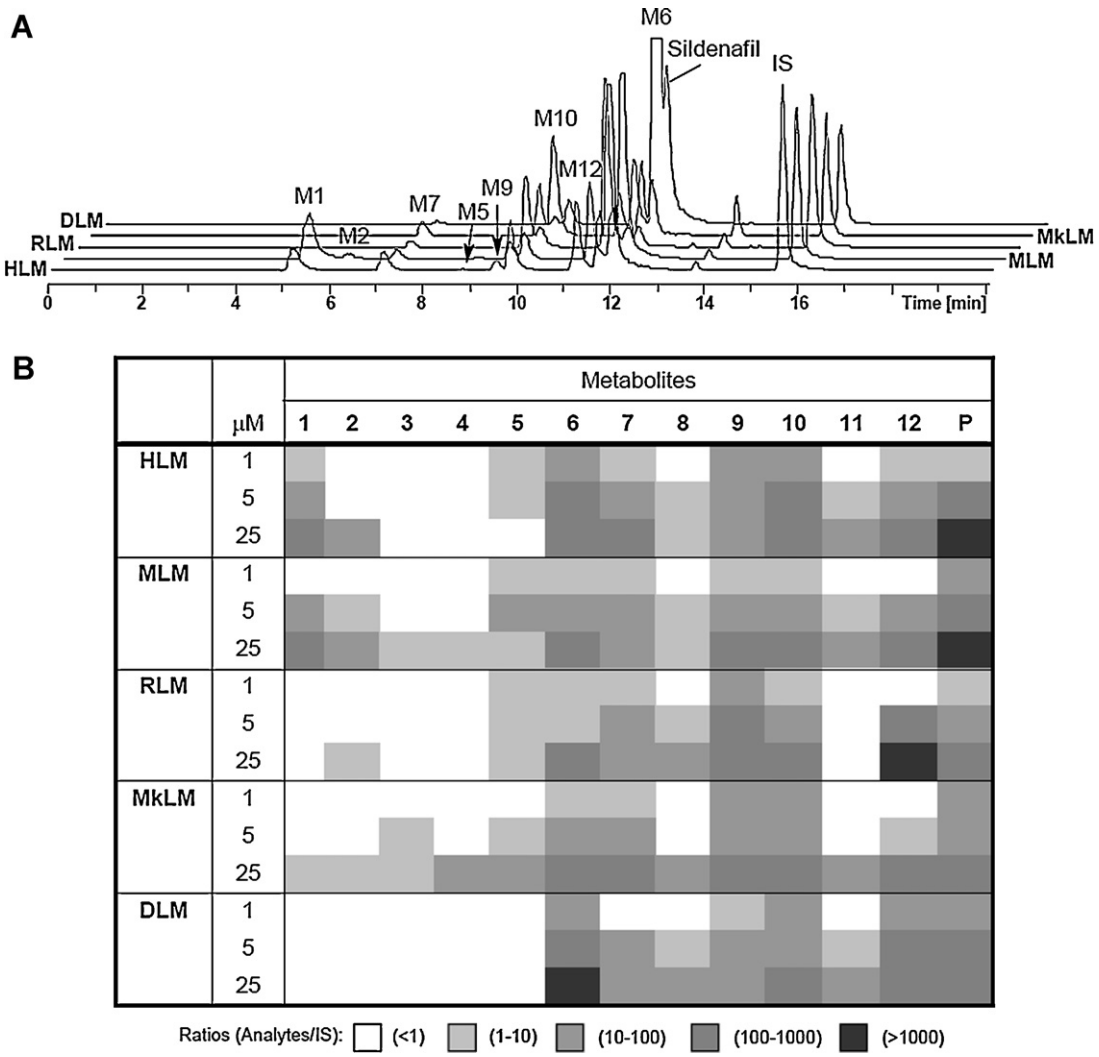

Fig. 4. Representative overlaid total ion chromatograms (at 25 μM of sildenafil) (A) and peak area map plotting the peak-area ratios of analytes versus the internal standard (B) for sildenafil and its potential metabolites following microsomal incubation of sildenafil. HLM, human liver microsomes; MLM, mouse liver microsomes; RLM, rat liver microsomes; MkLM, monkey liver microsomes; DLM, dog liver microsomes.

by piperazine *N*,*N*-deethylation from M10 and M12; M8, at *m/z* 447, by *N*-didemethylation on the pyrazole and piperazine moiety from M11 and M12; and M7, at *m/z* 377, by loss of the piperazine ring. Almost all of the metabolites, except M7, found in this study were found in previous studies [15]. The elemental composition analysis and collision-induced dissociation fragmentation pattern (Fig. 2) indicated that M7 is generated from the loss of the piperazine ring. M7 was found in all species investigated (Supplemental Fig. S1) and this metabolic pathway was also found after the metabolism of mirodenafil, an analog of sildenafil [17]. In addition, more hydroxylated metabolites were found in this study than in the previous study [15]. The postulated metabolic pathway is presented in Fig. 3.

3.2. Pattern recognition analysis using PCA

The overlaid total ion chromatogram and the corresponding peak area map for the sildenafil metabolic profile in different species are shown in Fig. 4. Based on the chromatographic data for each species, PCA was conducted. According to the PCA results, the first 3 PCs, PC1, PC2, and PC3, showed more than 88.6% of the total variability (data not shown). The PC score plots showed distinctive and isolated clusters for each species, indicating a characteristic interspecies variation for sildenafil metabolism (Fig. 5A). At a concentration of 1 μM sildenafil, the HLM group showed positive scores on all of PCs, i.e., PC1, PC2, and PC3, and was distinct from all the other groups. At 10 μM, HLM, MLM, and MkLM groups were clustered together, whereas RLM and DLM groups were located apart from the above-mentioned groups on PC2 and PC3. At 25 μM, the HLM group characterized by negative scores on PC2 was clustered with the MLM group and separated from the other groups that had positive scores on PC2. These findings suggest the potential similarity in the metabolic profiles of mice and monkeys with that of humans.

The loading plots presenting loading values of the variables associated with the first 3 PCs, i.e., PC1, PC2, and PC3, are shown in Fig. 5B. From the loading plot, M10 may be a characteristic metabolite present at a low concentration (1 μM) in humans; the peak of the parent drug (sildenafil) was the most contributive factor in characterizing its metabolism in humans as its concentration increases. M6 and M9 were found to be distinctive metabolites that distinguished the metabolism patterns in dogs and rats from those in humans.

3.3. SI measurement between human and other species

The SI values of the metabolism patterns between humans and other species were calculated using the Bray–Curtis equation (Table 2). At a low concentration of sildenafil (1 μM), all species

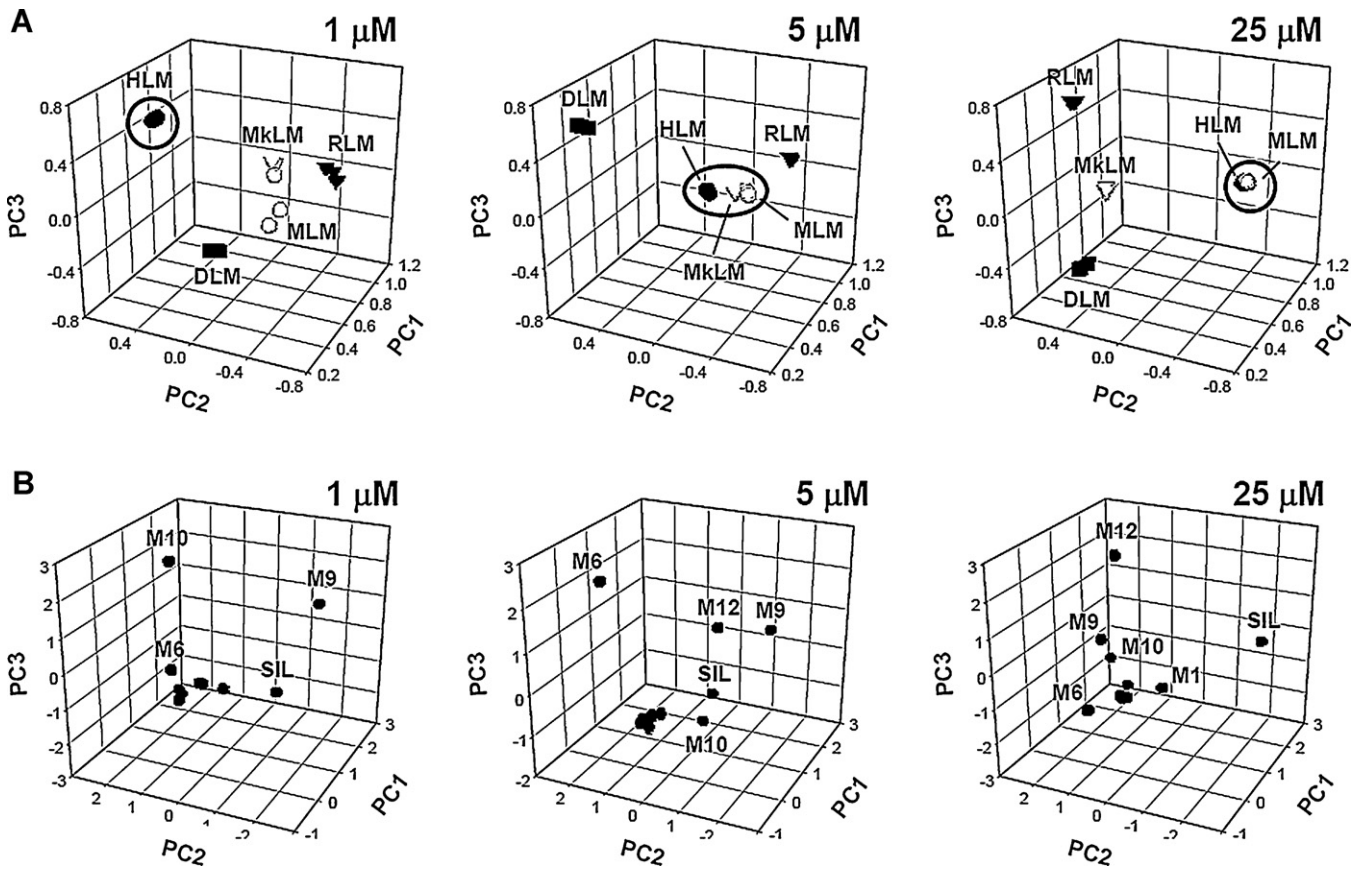

Fig. 5. PC score plots (A) and loading plots (B) for metabolism profiles of sildenafil in liver microsomes from 5 species.

Table 2
SI index between sildenafil metabolism patterns of a human and other species.

Species	The concentration of sildenafil (μM)		
	1	5	25
Mouse	0.390 ± 0.031[b]	0.698 ± 0.046[a]	0.764 ± 0.070[a]
Rat	0.401 ± 0.016[b]	0.420 ± 0.009[c]	0.340 ± 0.004[c]
Monkey	0.509 ± 0.013[a]	0.491 ± 0.021[b,c]	0.504 ± 0.010[b]
Dog	0.396 ± 0.003[b]	0.510 ± 0.014[b]	0.422 ± 0.005[b,c]

Each value represents the mean ± S.E. of triplicate tests. Duncan's *t*-test was used to investigate statistical significance of data. The significant values at $P < 0.05$ were represented as different alphabets.

had low SI values, i.e., less than 0.5. However, mice showed a pattern similar to humans as the concentration increased and were the closest to humans in terms of sildenafil metabolism (SI = 0.764). The SI values of the other species were low at the concentrations tested. Generally, the SI results were comparable to the PCA results.

4. Discussion

In this study, we attempted to use chemometric approaches i.e., PCA, a type of pattern-recognition analysis, and SI measurement, to investigate interspecies differences in sildenafil metabolism. The metabolic profile at 3 concentration levels of sildenafil (1, 5, and 25 μM) was characterized and applied to PCA and SI measurement. The concentrations investigated in the present study were chosen on the basis of the K_m value of sildenafil for the major metabolic reaction and its toxic concentration. Sildenafil is known to be metabolized by CYPs to yield an *N*-demethylated metabolite, UK-103,320 (M12), as the main circulating metabolite [15,18]. According to a report by Warrington et al. [16], in human liver microsomes, the K_m of sildenafil for the formation of UK-103,320 was 14.4 μM. Therefore, a concentration range that includes the lower and higher values than the K_m value was chosen so that possible changes in the metabolic profile after metabolic saturation could be observed. In addition, 30 μM of sildenafil is known to prolong cardiac repolarization, which is relevant to cardiac toxicity [19,20]. The highest concentration tested in this study (25 μM), which is close to the toxic concentration, reflects the metabolic profile under the conditions in which the drug is toxic.

The PCA results for the metabolism profile of sildenafil are represented by the scores and loading plots (Fig. 5). The PC score plot shows the interspecies variation in sildenafil metabolism at different concentrations. As the concentration changed, the pattern of PC scores for each species also changed. At a low concentration of sildenafil (1 μM), the metabolism in humans was not similar to that in the other species. However, the data points for humans gradually shifted positively on the PC2 axis as the concentration increased and clustered with the data points of MkLM and MLM at 5 μM and 25 μM, respectively. Considering the K_m of sildenafil for the major metabolic reaction (14.4 μM), these changes in the PC pattern are thought to be attributed to metabolic saturation of sildenafil in HLM. This is supported by the peak area map (Fig. 2B), where sildenafil gradually occupied a larger portion, depending on the concentration. The loading plot showed the variables responsible for the results of the score plot. The results of the loading plot showed that M1, M6, M9, M10, M12, and the parent drug sildenafil considerably contributed to the characterization of the metabolic profile of each species on the basis of the PC scores. The high sildenafil content was a decisive factor characterizing the metabolic profile of sildenafil in humans at toxic concentrations.

Furthermore, a similarity analysis based on matching of datasets with the human dataset was performed for the other species. The

similarity analysis scores indicate the extent of similarity between 2 different datasets as SI values and provide clearer information while various datasets are compared. For example, it could be used to select an experimental animal that has a metabolism pattern closer to that of humans. The results of this study show that mice had the highest SI value among the species tested, and this finding is consistent with the results of the PCA.

In nonclinical safety studies, suitable animal models for toxicology studies should have metabolic profiles comparable to that of humans [21]. Thus, it is favorable to select an animal species with an equivalent or similar metabolic pattern to that of humans. However, the comparison of metabolic profiles between animals and humans is sometimes ambiguous when drugs possess a complex pattern of metabolism (Fig. 4). Thus, a multivariate statistical approach could be used to investigate the comparative metabolism of a xenobiotic and suggest animal models for preclinical toxicology studies. In the case of sildenafil, mouse and monkey could be chosen as the toxicology species on the basis of PCA and SI. Further, their metabolic profiles reflected all metabolites produced in humans, including the active metabolite M12. Use of various weighting processes before PC or SI analysis [22], particularly positive or negative weighting to specific metabolites such as active/toxic metabolites and missing/superfluous metabolites, in certain species could provide highly accurate results in the assessment of metabolic behavior.

5. Conclusion

In conclusion, a study of interspecies differences in the metabolism of sildenafil was conducted using an LC/MS analysis combined with multivariate analysis approaches such as PCA and similarity analysis. This approach allowed us to elucidate the similarity of metabolic patterns of different species, which may be a useful tool to evaluate the comparative metabolism of xenobiotics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.08.037.

References

[1] R. Ramanathan, S.N. Çömezoğlu, W.G. Humphreys, in: W.A. Korfmacher (Ed.), Using Mass Spectrometry for Drug Metabolism Studies, CRC Press, Boca Raton, 2010, p. 128.
[2] S.A. Wrighton, B.J. Ring, M. VandenBranden, Toxicol. Pathol. 23 (1995) 199.
[3] S. Ekins, B.J. Ring, J. Grace, D.J. McRobie-Belle, S.A. Wrighton, J. Pharmacol. Toxicol. Methods 44 (2000) 313.
[4] A. Rostami-Hodiegan, G.T. Tucker, Nat. Rev. Drug Discov. 6 (2007) 140.
[5] J. Sahi, S. Grepper, C. Smith, Curr. Drug Discov. Technol. 7 (2010) 188.
[6] J.L. Bussiere, Expert Opin. Drug Metab. Toxicol. 4 (2008) 871.
[7] R.S. Plumb, C.L. Stumpf, M.V. Gorenstein, J.M. Castro-Perez, G.J. Dear, M. Anthony, B.C. Sweatman, S.C. Connor, J.N. Haselden, Rapid Commun. Mass Spectrom. 16 (2002) 1991.
[8] G.L.A.H. Jones, E. Sang, C. Goddard, R.J. Mortishire-Smith, B.C. Sweatman, J.N. Haselden, K. Davies, A.A. Grace, K. Clarke, J.L. Griffin, J. Biol. Chem. 280 (2005) 7530.
[9] S. La, H.H. Yoo, D.H. Kim, Chem. Res. Toxicol. 18 (2005) 1887.
[10] F. Li, X. Lu, H. Liu, M. Liu, Z. Xiong, Biomed. Chromatogr. 21 (2007) 397.
[11] X.L. Piao, J.H. Park, J. Cui, D.H. Kim, H.H. Yoo, J. Pharm. Biomed. Anal. 44 (2007) 1163.
[12] J.R. Bray, J.T. Curtis, Ecol. Monogr. 27 (1957) 325.
[13] K.R. Clarke, Aust. J. Ecol. 18 (1998) 117.
[14] M. Boolell, M.J. Allen, S.A. Ballard, S. Gepi-Attee, G.J. Muirhead, A.M. Naylor, I.H. Osterloh, C. Gingell, Int. J. Impot. Res. 8 (1996) 47.
[15] D.A. Walker, M.J. Ackland, G.C. James, G.J. Muirhead, D.J. Rance, P. Wastall, P.A. Wright, Xenobiotica 29 (1999) 297.
[16] J.S. Warrington, R.I. Shader, L.L. von Moltke, D.J. Greenblatt, Drug Metab. Dispos. 28 (2000) 392.
[17] J. Lee, H.H. Yoo, K.J. Rhim, D.R. Sohn, D.H. Kim, Rapid Commun. Mass Spectrom. 21 (2007) 1139.
[18] J.D. Cooper, D.C. Muirhead, J.E. Taylor, P.R. Baker, J. Chromatogr. B 701 (1997) 87.
[19] D. Abbott, P. Comby, C. Charuel, P. Graepel, G. Hanton, B. Leblanc, A. Lodola, L. Longeart, G. Paulus, C. Peters, J. Stadler, Int. J. Impot. Res. 16 (2004) 498.
[20] P. Geelen, B. Drolet, J. Rail, J. Bérubé, P. Daleau, G. Rousseau, R. Cardinal, G.E. O'Hara, J. Turgeon, Circulation 102 (2000) 275.
[21] M3(R2), Nonclinical safety studies for the conduct of human clinical trials and marking authorization for pharmaceuticals. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073246.pdf (accessed 9.01.10), part of Guidance for Industry. http://www.fda.gov/cder//guidance.
[22] H.C. Keun, T.M. Ebbels, H. Antti, M.E. Bollard, O. Beckonert, G. Schlotterbeck, H. Senn, U. Niederhauser, E. HolmesE, J.C. Lindon, J.K. Nicholson, Chem. Res. Toxicol. 15 (2002) 1380.