Original Article

DOI: 10.5582/ddt.2021.01101

Quantification of antipsychotic biotransformation in brain microvascular endothelial cells by using untargeted metabolomics

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SUMMARY Most studies of antipsychotic-therapies have highlighted the discrepancy between plasma and brain pharmacokinetics of antipsychotics, but how the drug changes through the blood brain barrier (BBB) has not been investigated. Cell-based metabolomics using liquid chromatography-mass spectrometry (LC-MS) combined with multivariate data analysis were applied for screening of antipsychotic metabolites in the BBB. We applied this approach to analyze the antipsychotic biotransformation in brain microvascular endothelia cells (BMVECs), the main component of the BBB. From this study, five, four, three, and one metabolite of chlorpromazine, clozapine, haloperidol and risperidone, respectively, were locally metabolized on the BMVECs. These results confirm that there is a drug biotransformation process within the BBB and show that drug metabolite screening employed cell-based metabolomics using LC-MS, combined with multivariate analysis in the study of BMVECs exposed to antipsychotics can provide a way to screen drug metabolites in the BBB.

Keywords Biotransformation, antipsychotics, BBB, BMVECs, cell-based metabolomics, LC-MS, multivariate data analysis

1. Introduction

Antipsychotics have been widely used in treating mental health illnesses such as schizophrenia and bipolar disorder (*1*). Antipsychotics are normally classified as typical (such as chlorpromazine and haloperidol) or atypical (such as risperidone and clozapine). Clinical research on how to best predict the therapeutic effects and side effects of antipsychotics has been ongoing for several decades (*2-4*). Most studies have highlighted the studies on the pharmacokinetics and pharmacodynamics in brain tissue, cerebrospinal fluid and interstitial fluid for drugs used in the treatment of antipsychotics (*5,6*), but how the drug biotransformation through the blood brain barrier (BBB) has not been studied.

The BBB is a complex vascular structure that physically and physiologically separates the peripheral blood circulation from the central nervous system (CNS). It acts very effectively in maintaining brain homeostasis, regulating the influx and efflux transport of nutrients, and protecting the CNS from pathogens and toxins (*7*). The basic anatomy of the BBB consists of brain microvascular endothelial cells (BMVECs), pericytes, astrocyte foot processes and nerve endings. Although, this structure contributes to the function of the microvasculature in the brain, the permeability of the BBB is controlled only by the BMVECs (*7*).

The combined surface area of BMVECs constitutes by far the largest surface area for blood-brain exchange. This surface area, depending on the anatomical region, is between 150 and 200 cm^2/g of tissue giving a total area for exchange in the brain of 12-18 m^2 for the average human adult (*8*). BMVECs are the major site of blood-central nervous system (CNS) exchange and shield the brain against drugs, toxins and immune cells *via* paracellular, transcellular, transporter, and extracellular matrix proteins (*7,8*). While evidence for drug biotransformation exists in the BBB, has not investigated, and whether BMVECs themselves are functionally compromised metabolism and lead to the clinical response to drugs is unclear.

Cell-based metabolomics is the comprehensive analysis of small molecule metabolites in cell cultures by the integration of state-of-art analytical tools and bioinformatics (*9,10*). At present, liquid chromatography-mass spectrometry (LC-MS) as an analytical platform is quite commonly used in cell-based metabolomics. This method offers advantages over other analytical platforms; these include speed, sensitivity, relative ease of sample preparation and large dynamic range (*11*). Multivariate

data analysis, such as principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), is an essential component in cell-based metabolomics analysis, to assist in the extraction of valuable information from large LC-MS datasets (*12*). PCA statistical analysis is commonly employed to analyze multivariate data, due to its rapid provision of an overview of the information hidden in the LC -MS data (*13*). The OPLS-DA model is intended for the modeling of two classes of LC-MS data in order to improve class separation, simplify interpretation and identify potential biomarkers (*14*). The advantages of LC-MS, coupled with multivariate data analysis, mean they have been widely used in various fields, such as toxicology, pharmacology and medicine (*10,11,15*). Cell-based metabolomics using LC-MS coupled with multivariate data analysis has also been adopted in drug metabolism and is used for the screening of stable metabolites and reactive metabolites (*16*).

In this study, the implications of the cell-based metabolomics using LC-MS, coupled with multivariate data analysis, the in profiling of antipsychotic metabolism and bioactivation were firstly provided in BMVECs.

2. Materials and Methods

2.1. Materials

Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=87*) provides the details of the chemicals used in this study.

2.2. Cell lines and culture

For the current study, the brain microvascular endothelial cells, a fundamental of the BBB, were provided by Paul A. Smith (School of Life Science, University of Nottingham Medical School, Nottingham, UK). The BMVECs were used from passage 21-23 and were cultured and maintained as previously described in Elmorsy *et al.* (2004) (*17*).

2.3. Cytotoxicity assay

For this assay, the cell proliferation kit I (Merck, Bangkok, Thailand) was used to analyze the number of viable BMVECs. This kit is based on a colorimetric assay that analyzes the number of cell viable cells by the cleavage of tetrazolium salts (MTT) added to the culture medium. Briefly, BMVECs were seeded at 1 × 10^4 cells per well in 96-well plastic plates (Gibthai, Bangkok, Thailand) and incubated overnight at 37°C under humidified 5% CO_2 conditions. To assess the cytotoxicity of antipsychotics used were then incubated for 24 h. in the presence of drug or its vehicle (ethanol). The antipsychotics concentrations used in this study were 0.2 µM chlorpromazine (*18*), 1 µM clozapine (*19*), 0.7 µM haloperidol (*20*), and 0.5 µM risperidone (*21*); these concentrations are similar to those measurement in patients. The original drug and vehicle were corrected *via* subtraction of a blank (media with the kit reagents but without BMVECs).

2.4. Antipsychotic metabolite study

BMVECs were used at a density of 1 × 10^8 cell/mL and were cultured and maintained as described in the previous protocol (*17*). When 80% confluence had been achieved, unattached BMVECs and cell culture media were discarded and attached BMVECs were washed with 5 mL of phosphate buffer solution (PBS) (Merck, Bangkok, Thailand). Then, 10 mL of fresh cell culture media (Gibthai, Bangkok, Thailand) was added, followed by antipsychotics. The concentration of each drug was described in the previous section. This solution was then incubated for 24 h, then the cell culture media was removed, and attached BMVECs were washed with 5.0 mL of PBS followed by 0.5 mL of cold methanol (4oC). Then a cell scraper was used to detach cells from a flask and the cell suspension was transferred to an Eppendorf tube and kept at -80°C for further processes.

2.5. Drug metabolite extraction

The cell extraction was based on the method described by the previous paper (*22*). Briefly, 0.50 mL of 4°C chloroform and 0.50 mL of 4°C water were added to the suspension and then vortexed vigorously for 10 min at 4°C. Then, the suspension was centrifuged at 15,000 g for 10 min at 4°C. The hydrophilic fraction and hydrophobic fraction were collected separately and transferred into fresh Eppendorf tubes and evaporated to dryness at room temperature. The dried hydrophobic layer was reconstituted in 50 µL of chloroform and methanol (1:2, v/v), and the dried hydrophilic layer was reconstituted in 50 µL of water. The reconstituted samples were centrifuged at 15,000 g for 10 min at 4°C to remove any cell debris. Finally, the supernatant was transferred into an HPLC vial and stored at -80°C for LC-MS analysis.

2.6. LC-MS analysis

Metabolic profiling was performed on an LC Accela™ system (Thermo Scientific Ltd., Loughborough, UK) coupled with high resolution mass spectrometry (Exactive®, Thermo Scientific Ltd., Loughborough, UK). Hydrophobic chromatographic separations were performed on an Agilent SB C8 column (1.8 µm particle size, 2.1 × 100 mm, Crawford Scientific Ltd., Lanarkshire, UK) and hydrophilic chromatographic separations were performed on a C18 (2) column (2.5 µm particle size, 3 × 100 mm, Phenomenex Ltd, Cheshire, UK). The details of LC and MS conditions are

summarized in Supplementary Tables S2 and S3 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=87*), respectively. In both hydrophobic and hydrophilic metabolic profiling, samples were performed in six replicates, to account for any biological variability. The retention time consistency and mass accuracy were confirmed though the pooled samples.

2.7. Data analysis

The feasibility of the method was first performed using a high mass resolution mass spectrometer. Six replicates of each drug-treated sample were analyzed by LC-MS. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were employed to process the acquired LC-MS data. Samples were grouped together for OPLS-DA modelling. The PCA and OPLS-DA results were displayed as score plots to visualize sample clustering and to indicate sample similarity. Discriminatory metabolites between the treated and the control for each antipsychotic drug were first screened with the variable importance in the projection (VIP) ranks > 1.00 and then validated using ANOVA statistical analysis of false discovery rate (FDR) with a significance level of 0.05 for antipsychotic treatments. According to the identity check, based on raw data and the features of peaks, the target masses of candidate metabolites identified in the profiling process were searched over a narrow ± 5 ppm mass window in the HMDB database and confirmed by available standards. The possible drug metabolite analysis combined results from the publications to help our studies identify the most relevant drug metabolites involved in the conditions under study. A results report was then presented graphically as well as in a detailed table.

3. Results

3.1. BMVEC viability

The viability results of cytotoxicity assay after incubation for 24 h with chlorpromazine, clozapine, haloperidol, risperidone, and vehicle (ethanol) were 99.7 ± 1.2%, 101.2 ± 0.7%, 100.5 ± 2.0%, 99.5 ± 1.3%, and 100.2 ± 0.3%, respectively. These results confirmed that the drug concentration used did not affect the cell growth.

3.2. LC-MS data quality

Figures 1 and 2 illustrate the LC-MS chromatograms

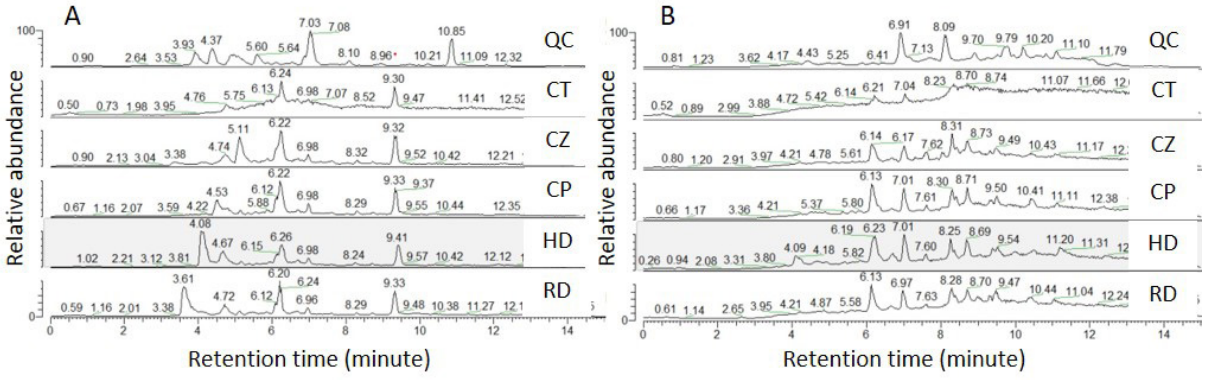

Figure 1. LC-MS chromatogram of hydrophobic fraction in ESI positive (A) and ESI negative (B) of pooled sample (QC), control sample (CT), chlorpromazine treatment (CZ), clozapine treatment (CP), haloperidol treatment (HD), and risperidone treatment (RD).

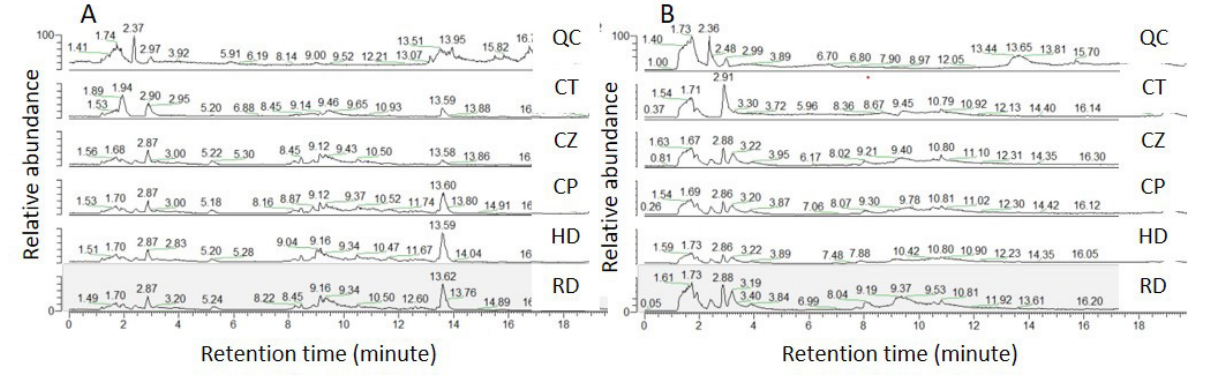

Figure 2. LC-MS chromatogram of hydrophilic fraction in ESI positive (A) and ESI negative (B) of pooled sample (QC), control sample (CT), chlorpromazine treatment (CZ), clozapine treatment (CP), haloperidol treatment (HD), and risperidone treatment (RD).

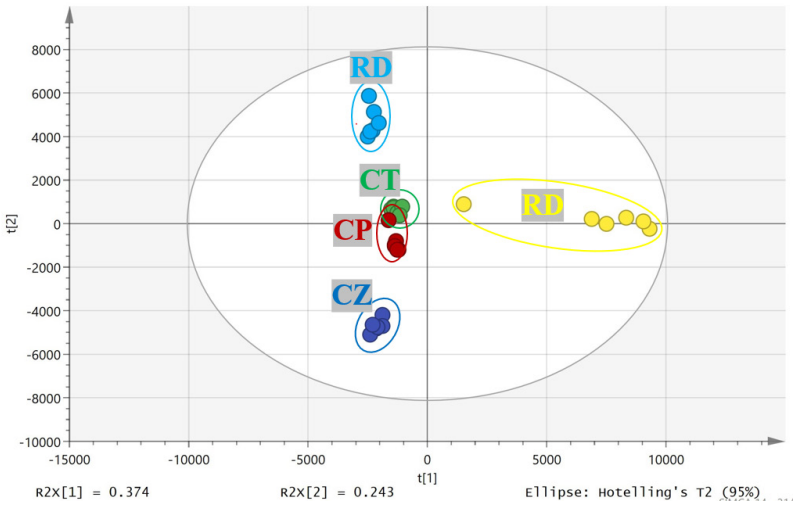

Figure 3. PCA scores plots of metabolites of brain microvascular endothelia cells treated with control sample (CT), chlorpromazine treatment (CZ), clozapine treatment (CP), haloperidol treatment (HD), and risperidone treatment (RD).

of hydrophobic and hydrophilic fractions extracted from BMVECs treated with antipsychotics in ESI positive and negative, respectively. To check the LC-MS instrument performance in the current study, 10.0 µL of cellular extraction from each BMVEC sample were pooled to get a quality control (QC) sample in both hydrophobic and hydrophilic fractions. Several consecutive injections of the QC sample were made to obtain a stable LC-MS system. One in five QC samples was analyzed throughout the whole analysis procedure for both hydrophobic and hydrophilic fractions.

According to the optimized conditions, principal component analysis (PCA) of all samples from BMVECs is shown in Figure 3. The QC samples were gathered together for analysis during the data collection. The data from the QC samples were then analyzed to determine the number of ions present in the samples, their intensity and their % RSD values. The average RSDs of peak abundance for the QC samples was 18.0% with a standard deviation of 7.8% for the hydrophobic part, and 22.9% with a standard deviation of 10.2% for the hydrophilic part. Since the recommendation of the FDA is that biochemical analysis ions should show RSDs of less than 30%, this recommendation was used in the subsequent analysis of the test and control sample data (*23*). The results demonstrated that the system employed in this study had excellent stability during the analysis procedure for both hydrophobic and hydrophilic part.

For data analysis, the aligned data array was filtered using the QC samples. In line with the recommendations of the FDA for biomarker analysis ions (*23*), those showing RSDs less than 30% were used in the subsequent analysis of the BMVECs treated with four antipsychotics and control cell sample data.

3.3. Cell metabolic profiling of BMVEC treated with four antipsychotics

From our data, PCA score plots showed clear separation between control and antipsychotic treatment of BMVECs (Figure 3). Figure 3 shows that separate clusters from each model are revealed, which indicates metabolic differences in terms of level and compositional changes of cellular metabolites among control, chlorpromazine treatment, clozapine treatment, haloperidol treatment and risperidone treatment. Then, OPLS-DA was applied to visualize samples in an attempt to distinguish between control and each antipsychotic treatment.

A very clear separation was revealed in Figures 4A-4D. In order to identify drug metabolites, VIP statistics (VIP > 1.0) were initially used to pre-select detected mass ions. Then, from those detected mass ions with FDR (ANOVA) < 0.05 selection was made of those which were most correlated highly with the OPLS-DA discriminant scores in order to decrease the risk of false positives in the selection of significantly altered mass ions.

3.4. Antipsychotic drug metabolism in BMVECs

For the LC-MS analysis of the four antipsychotic drugs mentioned above, samples obtained from incubation with BMVECs with chlorpromazine, clozapine, haloperidol, or risperidone were extracted by liquid extraction and concentrated by speed vacuum. The MS spectrum of the chlorpromazine $[M+H]^+$, haloperidol $[M+H]^+$, clozapine $[M+H]^+$, and risperidone $[M+H]^+$ revealed ions at m/z = 319.1028, 376.1473, 327.1327, and 411.2190, respectively. Of interest from the current perspective was the detection of each drug metabolism as shown in Table 1, while Figures 5-8 show possible chemical structures.

4. Discussion

Antipsychotic metabolite identification in BMVECs is challenging, since thousands of cellular metabolites

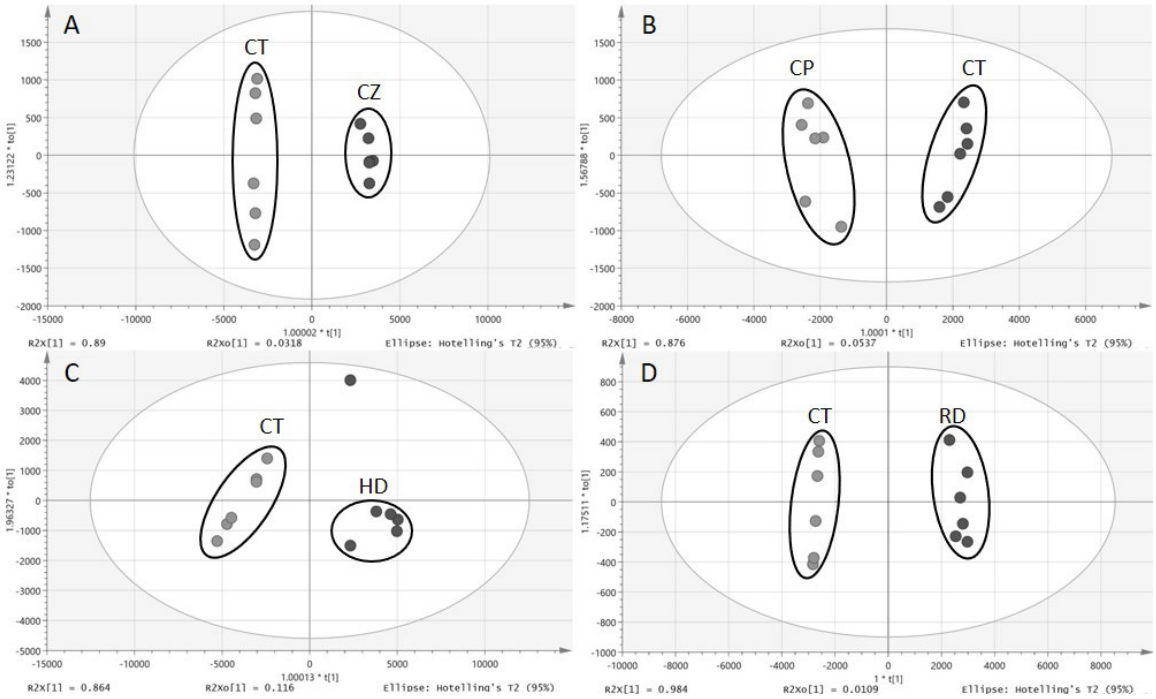

Figure 4. OPLS-DA of untargeted metabolomics data from BMVECs treated with antipsychotics. Two-dimensional OPLS-DA scores plots reveal separation between **(A)** the control group (CT) and the chlorpromazine-treated group (CZ), **(B)** the control group (CT) and the clozapine-treated group, **(C)** the control group (CT) and haloperidol-treated group (HD), **(D)** and the control group (CT) and the risperidone-treated group (RD).

Table 1. Possible biotransformation of chlorpromazine, haloperidol, clozapine, and risperidone by brain microvascular endothelial cells

Name	m/z	Adduct	RT (min.)	MW	Formula	VIP	FDR	Confirmation
Chlorpromazine	319.1028	$[M+H]^+$	5.08	318.0957	$C_{17}H_{19}ClN_2S$	1.85	3.53×10^{-5}	Standard
7-Hydroxy-chlorpromazine	355.0978	$[M+H]^+$	6.72	334.8636	$C_{17}H_{19}ClN_2OS$	2.01	9.12×10^{-6}	Standard
3-Hydroxy-chlorpromazine	355.0978	$[M+H]^+$	6.72	334.8636	$C_{17}H_{19}ClN_2OS$	2.02	4.28×10^{-6}	Standard
Norchlorpromazine	305.0872	$[M+H]^+$	4.69	304.8376	$C16H_{17}ClN_2S$	2.03	3.32×10^{-8}	Standard
Chlorpromazine-N-oxide	335.0978	$[M+H]^+$	3.37	334.0907	$C_{17}H_{19}ClN_2OS$	2.02	3.16×10^{-7}	Standard
Promazine	285.1419	$[M+H]^+$	4.53	284.4191	$C_{17}H_20N_2S$	2.02	2.84×10^{-6}	Standard
Haloperidol	376.1473	$[M+H]^+$	4.06	375.8642	$C_{21}H_{23}CLFNO_2$	1.98	1.62×10^{-6}	Standard
Haloperidol-N-oxide	392.1421	$[M+H]^+$	3.27	391.8636	$C_{21}H_{23}CLFNO_3$	2.03	3.43×10^{-7}	Standard
Reduced haloperidol	378.1631	$[M+H]^+$	4.34	377.8801	$C_{21}H_{25}CLFNO_2$	2.04	8.91×10^{-7}	Standard
Dechloro haloperidol	342.1863	$[M+H]^+$	3.61	341.1492	$C_{21}H_{24}FNO_2$	2.03	6.00×10^{-6}	Standard
Clozapine	327.1327	$[M+H]^+$	4.46	326.8233	$C18H_{19}ClN_4$	1.97	1.71×10^{-4}	Standard
Hydroxy-clozapine	343.1319	$[M+H]^+$	3.40	342.8227	$C18H_{19}ClN_4O$	2.00	4.86×10^{-6}	Mass error < 5 ppm
Clozapine-N-oxide	343.1318	$[M+H]^+$	6.41	342.8227	$C18H_{19}ClN_4O$	2.02	3.79×10^{-3}	Standard
N-Desmethylclozapine	313.1213	$[M+H]^+$	3.78	312.7967	$C_{17}H_{17}ClN_4$	2.03	1.80×10^{-3}	Standard
Hydroxyl-desmethyl-clozapine	307.1553	$[M+H]^+$	3.32	294.3510	$C_{17}H18N_4O$	2.04	5.48×10^{-4}	Mass error < 5 ppm
Risperidone	411.2190	$[M+H]^+$	3.61	410.4845	$C_{23}H_27FN_4O_2$	1.99	1.69×10^{-13}	Standard
Hydroxy-risperidone	427.2139	$[M+H]^+$	3.67	426.4839	$C_{23}H_27FN_4O_3$	2.04	6.77×10^{-6}	Standard

m/z: mass per charge ratio, RT: retention time, min: minute, MW = monoisotopic molecular weight, VIP: variable importance in the projection, FDR: false discovery rate and ppm: part per million.

exist in cells. Radiotracking is commonly utilized as a method for identifying the drug metabolites *in vivo* and *in vitro*. However, this method greatly depends on the availability of the radiolabeled molecules that are sometimes difficult and expensive to synthesize and require containment facilities. Moreover, radiolabeled molecules can be metabolized at different rates by enzymes. Accompanying the development of technology associated with metabolomics, several metabolomics-based methods have been developed, such as cell-based metabolomics using LC-MS, combined with multivariate data analysis for screening and characterizing drug metabolites. Moreover, this method is an unbiased approach for metabolite

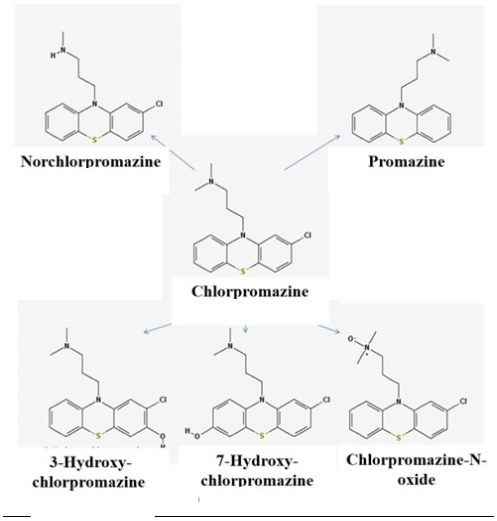

Figure 5. Schematic representation of the possible metabolism of chlorpromazine by brain microvascular endothelial cells.

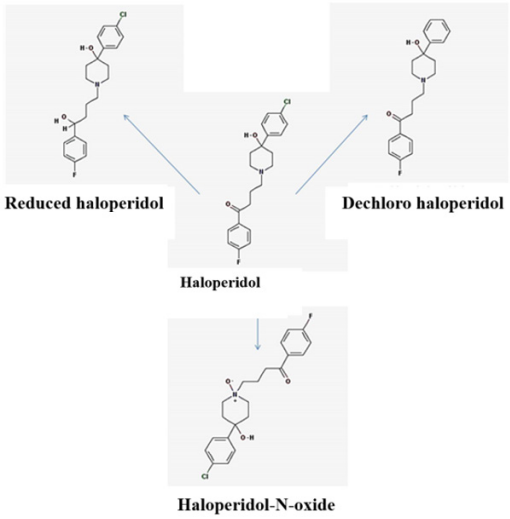

Figure 6. Schematic representation of the possible metabolism of haloperidol by brain microvascular endothelial cells.

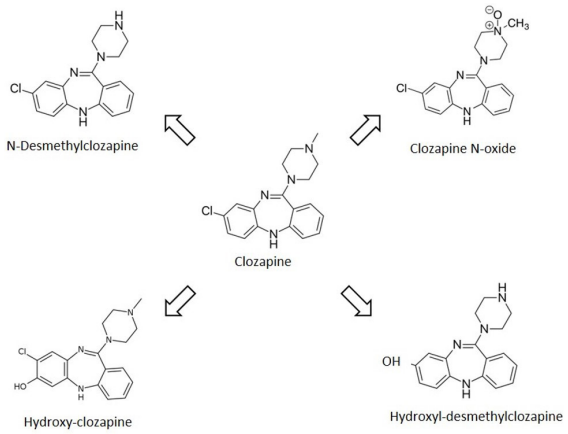

Figure 7. Schematic representation of the possible metabolism of clozapine by brain microvascular endothelial cells.

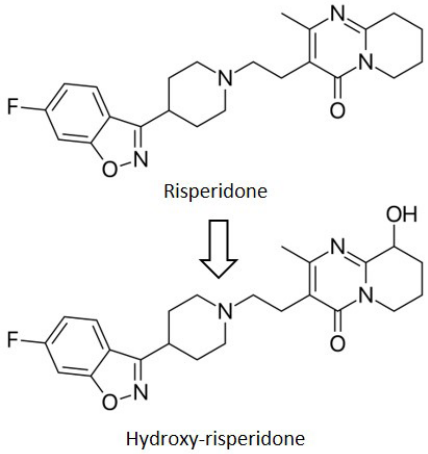

Figure 8. Schematic representation of the possible metabolism of risperidone by brain microvascular endothelial cells.

identification. The purpose of this study was to firstly describe the outcomes of cell-based metabolomics using LC-MS combined with multivariate data analysis in profiling of antipsychotic metabolism and bioactivation in BMVECs.

Although only five chlorpromazine metabolites were identified in this study, these metabolites were identified in human serum (*24,25*) and *in vitro* study (*26,27*). Previous studies had suggested that chlorpromazine could be transformed into other metabolites through hydroxylation, N-oxidation, demethylation and dechlorination. To achieve chlorpromazine metabolites, LC-MS analysis was applied to screen for these compounds. The ions were identified as the putative chlorpromazine metabolites, based on the exact m/z as shown in Table 1 and confirmed by standards. This *in vitro* study detected the two hydroxylation chlorpromazine metabolites (8-hydroxychlorpromazine and 3-hydroxychlorpromazine), one demethylation chlorpromazine metabolite (N-desmethyl chlorpromazine), one N-oxidation chlorpromazine metabolite (chlorpromazine-N-oxide), and one chlorination chlorpromazine metabolite (promazine).

Moreover, using LC-MS, Khelfi *et al.* (2018) (*28*) reported 14 haloperidol metabolites formed from an *in vitro* study of human liver microsomal incubation. In this study they found only 3 drug metabolites in BMVECs as shown in Table 1 and Figure 7. This illustrates the possible chemical structures of the detected metabolites: N-oxidation, hydroxylation, and dechlorination forms. These drug metabolites were similarly found in the previous study (*28*).

In addition, there were 12 clozapine metabolite forms of clozapine which were reported in previous studies (*29*). In our study, four clozapine metabolite forms were detected in the BMVECs treatment group: one oxidation, one hydroxylation, one dechlorination, and one oxidative dechlorination (as shown in Table

1 and Figure 7). 9-Hydroxy risperidone was detected for risperidone metabolism in the BMVECs, as shown in Table 1 and Figure 9, according to the previous experiment (*30*).

These results confirm that there is a drug biotransformation process at the BBB and show that drug metabolite screening employed cell-based metabolomics using LC-MS combined with multivariate analysis in the study of BMVECs exposed to antipsychotics could provide a way to use for screening of drug metabolites in the BBB.

Acknowledgements

I would like to thanks Paul A. Smith (School of Life Science, University of Nottingham Medical School, Queens Medical Centre, Nottinghamshire, NG7 2UH, UK) for providing us with BMVECs. The authors are also grateful to Mr. John Tucker, MA in Language Testing, University of Lancaster, for kind help in the English correction.

Funding: This work was supported by a grant from Navamindradhiraj University, Bangkok, Thailand

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received November 23, 2021; Revised December 21, 2021; Accepted December 26, 2021

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