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Determination of brain cytochrome P450 2E1 activity in rat with the probe of chlorzoxazone by liquid chromatography–mass spectrometry

Jian-Yuan Wu[a,b], Jiang Yue[c], Yu-Qi Feng[a,*]

[a] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), Department of Chemistry, Wuhan University, Wuhan 430072, China
[b] ZhongNan Hospital of Wuhan University, Wuhan 430071, China
[c] Department of Pharmacology, Wuhan University, Wuhan 430071, China

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ABSTRACT

A simple and sensitive method was developed for the determination of cytochrome P450 2E1 (CYP2E1) activity based on the liquid chromatography–mass spectrometry (LC–MS) analysis of 6-hydroxychlorzoxazone generated by 6-hydroxylation of chlorzoxazone under specific catalysis of CYP2E1. In the proposed method, 2-benzoxazolinone was chosen as internal standard and isopropyl ether was used as extraction solvent for sample preparation. The inter-day and intra-day precisions at low, medium and high concentrations of 6-hydroxychlorzoxazone were below 20.0%, and the LOD (S/N = 3) was 0.05 ng/mL. This method was applied to analyze the CYP2E1 activity of rat in different brain regions including frontal cortex (FC), cerebellum (CB), brain stem (BS), hippocampus (HC), striatum (ST), thalamus (TH), and olfactory bulb (OB). The results confirmed that chlorzoxazone was a suitable probe for the determination of CYP2E1 activity in brain regions and samples with low content of CYP2E1.

1. Introduction

Cytochrome P450 (CYP) is the superfamily of heme-containing mono-oxygenases and responsible for the biotransformation of pharmaceutics, toxins, carcinogens and endogenous compounds (e.g. hormones and steroids) [1,2]. Cytochrome P450 2E1 (CYP2E1) is an ethanol-metabolizing enzyme that mono-oxygenates more than 70 low-molecular hydrophobic compounds, as well as much larger endogenous fatty acid signaling molecules (e.g. arachidonic acid) [3]. CYP2E1 has been shown to produce free radicals independent of a ligand which can cause cell damage from lipid peroxidation and DNA strand breaks [4]. Thus, CYP2E1 has been considered as an important determinant of environmental, industrial toxins or carcinogens in the degree of sensitivity to the body [5,6].

Although CYPs are primarily expressed in liver, many CYP isoforms also exist in extrahepatic tissues, including the brain. CYP2E1 protein has been shown to be expressed in mammalian brain. In rat brain, CYP2E1 expression was observed in pyramidal cells of frontal cortex, pyramidal and polymorphic cell layer of hippocampus and glial cells in olfactory bulb and piriform cortex [7,8]. Brain contains a large amount of phospholipids that are rich in polyunsaturated fatty acids, which are liable to peroxidation by reactive oxygen species, besides the limited regenerative capacity of the brain [9]. Hence, the CYP2E1 catalytic activity in diverse brain regions is of interest and the determination of the CYP2E1 activity in different brain regions may be important from neurophysiological and/or neurotoxicological points of view.

Most of the studies have indicated that the levels of brain CYP2E1 mRNA and protein are expressed at extremely low levels [10–12]. It would be extremely necessary to have a specific assay to detect the catalytic activity of CYP2E1 in different brain regions. Up to now, N-nitrosodimethylamine (NDMA), p-nitrophenol (PNP) and chlorzoxazone (CLZ) are the most effective probes for monitoring the level of CYP2E1 [13]. The structure of each of these substrates and the position of hydroxylation are shown in Fig. 1. The measurements of PNP hydroxylation or NDMA demethylation by spectrophotometry are simple and rapid; however, these assays are limited by the sensitivity of the methods. In contrast, the 6-hydroxylation of CLZ is more sensitive for the determination of CYP2E1 activity [14]. Moreover, the 6-hydroxylation of CLZ which has been clinically used for many years as a long-acting central muscle relaxant could not only be applied to evaluate the level of CYP2E1 in vitro, but also can be used as a noninvasive probe in vivo [15]. Usually, the CYP2E1 activity was determined by the urinary excretion of 6-hydroxychlorzoxazone (OH-CLZ) and the plasma ratio of OH-CLZ to CLZ as a noninvasive probe in vivo [16], and the CYP2E1 activity is calculated by dividing the amount of OH-CLZ formed by incubation time and microsomal protein content (pmol min^{-1} mg^{-1}) in vitro [17]. Various methods have been

* Corresponding author. Tel.: +86 27 68755595; fax: +86 27 68755595.
E-mail address: yqfeng@whu.edu.cn (Y.-Q. Feng).

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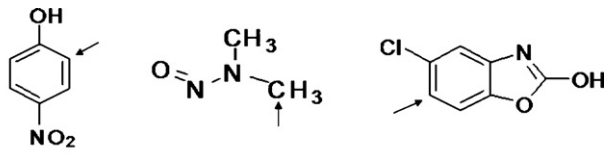

p-Nitrophenol (PNP) | N-Nitrosodimethylamine (NDMA) | Chlorzoxazone (CLZ)

Fig. 1. The structures of substrates probes to monitor the presence of CYP2El. The position of hydroxylation is indicated by the arrow for each structure.

reported for CLZ and OH-CLZ assay in biological fluids and microsomes, which included fluorimetry [16,18], gas chromatography with flame ionisation detection (GC–FID) [19], gas chromatography with mass spectrometry (GC–MS) [20], high performance liquid chromatography with UV detection (HPLC–UV) [21–30]. The analytical methods based on HPLC with UV detection have relatively poor detection sensitivity to measure both parent drug and metabolite, leading to a need for high dose levels of the parent drugs (in vivo) or more microsomal samples (in vitro). Meanwhile, the high-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) method was also reported [31–36]. This method was more sensitive and selective, but the reported papers only emphatically used for simultaneously monitoring several CYP-specific probe metabolites from human urine or plasma after single or N-in-one administration.

In this study, we describe an easy and sensitive LC–MS method for the determination of CLZ 6-hydroxylation in different rat brain regions. As brain CYPs are present in the membranes of microsomes, mitochrondria, lysosomes, peroxisomes, Golgi bodies as well as the outer plasma membrane surface [8], the whole membranes rather than microsomes from different brain regions were prepared for the analysis of CYP2E1 activity.

2. Experimental

2.1. Chemicals and materials

Chlorzoxazone (CLZ), 6-hydroxychlorzoxazone (OH-CLZ) and 2-benzoxazolinone (internal standard, IS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (HPLC-grade) were purchased from Fisher Company Inc. (Fairfield, OH, USA). Deionized distilled water was purified with a Milli-Q apparatus (Millipore, Bedford, MA, USA) system and was used for all aqueous solutions. All other reagents were of the highest purity commercially available.

Stock solutions of CLZ (1 mg/mL), OH-CLZ (1 mg/mL) and IS (0.1 mg/mL) were prepared in methanol and stored at 4 °C in the dark, solutions were stable for 6 months. Working solutions were made freshly from these stock solutions in distilled water.

2.2. Animals and brain regions

Adult, male Wistar rats (250–300 g) from Hubei Center for Disease Control and Prevention (Wuhan, China) were housed under standard breeding conditions. The rats were sacrificed by decapitation, and the brain was rapidly removed. The brain was dissected into seven regions, which are olfactory bulb (OB), striatum (ST), brainstem (BS), frontal cortex (FC), hippocampus (HC), cerebellum (CB), thalamus (TH). All dissections were performed according to the Paxinos and Watson stereotaxic atlas of the rat brain [37]. The seven regions of each brain were then taken within a few minutes and stored at −80 °C before the analysis.

2.3. Whole membrane preparation and protein assay

To ensure accurately measured CYP2E1 protein levels in each brain region, we used whole membrane preparations as reported [38]. Briefly, the rat brain tissues were homogenized manually in 100 mM Tris buffer containing 0.1 mM EDTA, 0.1 mM DTT and 0.32 M sucrose (pH 7.4). Then the homogenate was centrifuged at 3000× *g* for 10 min at 4 °C. The supernatant was removed and the pellet resuspended and centrifuged again at 3000× *g* for 10 min at 4 °C. The combined supernatant was then centrifuged at 110,000× *g* for 90 min at 4 °C and the pellet resuspended in 0.25 M sucrose. The membranes were aliquoted and stored at −80 °C before use. The protein content of brain membranes was assayed with the Bradford technique [39] using a Bio-Rad Protein Assay kit with BSA as a protein standard.

2.4. The 6-hydroxylation of CLZ catalyzed by CYP2E1 of brain membrane

Rat brain membrane containing 0.5 mg of proteins was added 0.05 mL of 2.5 mM CLZ, 0.01 mL 1 mM magnesium chloride, 0.1 mL NADPH generating system solution (contained 0.1 M isocitric acid, 4 mM NADP and 6 U isocitric acid dehydrogenase) and artificial cerebrospinal fluid solution (ACSF: 126 mM NaCl, 2.68 mM KCl, 1 mM Na_2HPO_4, 0.88 mM $MgSO_4$, 22 mM $NaHCO_3$, 1.45 mM $CaCl_2$, 11 mM D-glucose, pH 7.4) [40]. Samples were incubated for 90 min at 37 °C in a shaking water bath. The reaction was stopped by addition of 0.1 mL zinc sulphate (30%, w/v) and vigorously homogenized for 15 s. Then, each sample was spiked with 20 μL IS working solution (1.0 μg/mL) and homogenized. The solutions were stored at −20 °C until sample treatment and LC–MS detection.

2.5. Sample treatment

The samples were unfreezed and vortexed for 30 s, 4 mL isopropyl ether were added and vigorously vortexed for 3 min. Then the mixture was centrifuged (10 min, 4 °C, 10,000 rpm, Anke TGL-16G, Anting Scientific Instrument Factory, Shanghai, China) and the upper organic phase (3.6 mL) was transferred to a microcentrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 100 μL 20% acetonitrile (v/v), vortexed, and centrifuged at 16,000 rpm for 5 min, (TG16-W, Xianglu Centrifuge Instrument Co. Ltd., Changsha, China) again. The supernatant was then transferred to vials and 20 μL was injected onto the column for LC–MS analysis.

2.6. LC–MS and operating condition

A HPLC-ESI-MS system (Shimadzu LCMS-2010EV, Tokyo, Japan) was used for determination of the generated OH-CLZ. Data acquisition and processing were performed with the LC–MS solution workstation (Shimadzu, Version 3.50). The separation was performed on a Shim-pack VP-ODS column (Shimadzu, 150 mm × 2.0 mm I.D., 5 μm) at 40 °C. A phenomenex-C18 analytical guard cartridge (4 mm × 3.0 mm, Phenomenex Inc., CA, USA) was used. A mixture of (A) 100% acetonitrile and (B) distilled water was used as the mobile phase at a flow rate of 0.2 mL/min. The binary gradient profile for mobile phase was as follows: t_0, 20% A; t_{10}, 60% A; t_{15}, 20% A; t_{20}, 20% A (where t refers to time (min)). The system was ready for the next injection without further equilibration. The sample injection volume was 20 μL. Negative mode electrospray ionization and selected ion monitoring (SIM) were performed to simultaneously monitor ions at m/z 168, 170; 184, 186 and 134, which corresponded to the deprotonated molecular ions of CLZ, OH-CLZ and BZX (IS). Capillary voltage was 4.5 kV. Curved desolvation line (CDL) and heat block temperatures for the

analysis were set at 250 and 200 °C respectively. Drying and nebulizer gases of nitrogen were set at 1.5 L/min with a pressure of 0.04 MPa. The detector voltage was set at 1.4 eV.

2.7. Preparation of calibration standards and quality control samples

For matrix-matched calibration curves, the solution was prepared by transferring appropriate volumes of the OH-CLZ working solution to a clean vial containing rat brain membranes (whole rat brain prepared, 0.5 mg of proteins), and were not incubated but immediately treated with 30% zinc sulphate and spiked with 20 μL IS working solution (1.0 μg/mL). The following series of membranes standards were prepared: 0, 0.5, 1.0, 3.0, 5.0, 10.0 and 20.0 ng/mL of OH-CLZ. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area of OH-CLZ/peak area of IS) versus concentration, and fitted to the equation $Y = aX + b$ by linear regression. Quality controls (QC) samples at three different concentration levels (0.5, 3.0 and 20.0 ng/mL) were prepared separately in duplicate for method validation. The samples were processed as described in Section 2.5.

3. Results and discussion

3.1. Optimization of LC–MS conditions

CLZ, OH-CLZ and 2-benzoxazolinone (IS) are compounds with medium polarity, so the electrospray ionization (ESI) in negative ion mode was adopted for the MS analysis. Firstly, the analytical performance of the optimized LC–ESI-MS was determined by standard solution. The mass spectrum of CLZ, OH-CLZ and IS obtained in the full scan mode are shown in Fig. 2. The three compounds all responded best to negative ionization and the deprotonated molecular ions $[M-H]^-$ were present as major peaks. The target compounds were identified based on the retention time, presence and relative abundances of the *m*/*z* 186,184 for OH-CLZ and the *m*/*z* 168,170 for OH-CLZ corresponding to the characteristic isotopic of the cluster of the two chlorine atoms respectively. Subsequently, the generated OH-CLZ was quantified by selecting the parent ion at *m*/*z* 184 because of high sensitivity and no interference peak at the retention time of OH-CLZ. In each chromatographic run, five ions (*m*/*z* 186,184 for OH-CLZ, *m*/*z* 168,170 for CLZ and *m*/*z* 134 for IS) were monitored.

The reported HPLC separation for CLZ and OH-CLZ mostly employed the isometric condition, while there are distinct differences of the retention times due to their polarity. In order to improve the separation and shorten the run time, the gradient elution was used in this study. Usually, the formic acid, triflouroacetic acid, or acetic acid is used to improve the chromatographic peak shape and to provide a source of protons in reversed-phase LC–MS. In our work we use acetonitrile as organic modifier, and did not add any acid to the mobile phase due to the suppression of MS ionization in negative ion mode. Under the optimum assay conditions, CLZ, OH-CLZ and IS were well separated. Figs. 3 and 4 showed typical chromatograms obtained with blank, standard samples and different brain regions membranes of rat after 90 min incubation with CLZ. The retention times for CLZ, OH-CLZ, and IS were 10.16, 5.88, and 7.37 min respectively.

3.2. Selection of extraction solvents

The liquid–liquid extraction (LLE) [23,25–30] and solid-phase extraction (SPE) [21] methods have been used to extract CLZ and its metabolite OH-CLZ from biological samples. The SPE method was sensitive, but the cost associated with use of solid-phase extraction columns decreases the overall usability of this method [26].

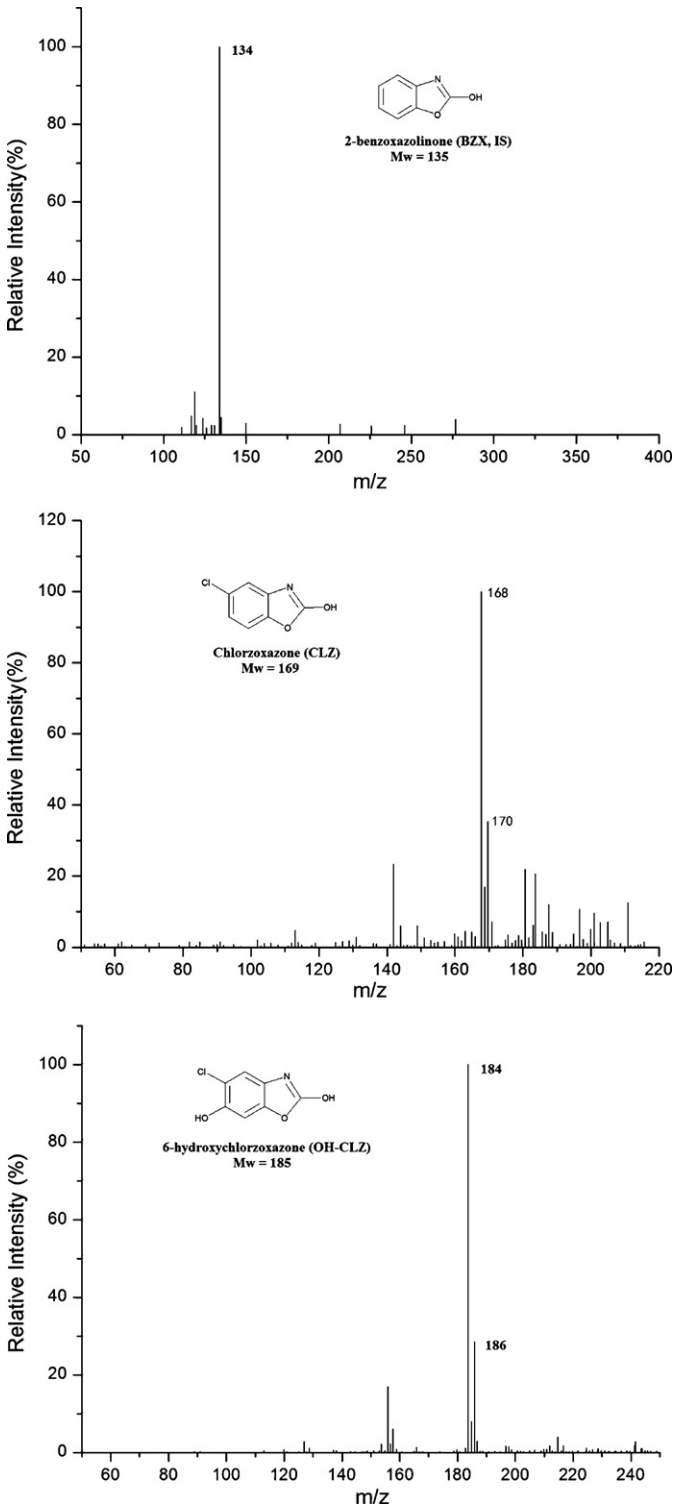

Fig. 2. Typical spectrum of OH-CLZ, CLZ and IS analyzed by LC–MS in the full scan mode, obtained in ESI negative ionization, also depicting their structure.

For some literatures, the incubated membrane solutions were centrifuged, filtered with a filter and direct injected onto the HPLC column for analysis [24,25]; however, these methods were poor in sensitivity and could not meet the requirement of low concentration of target compounds in samples. Organic solvents, such as chloroform, ether, ethyl acetate and isopropyl ether were tested in our study for solvent extraction of OH-CLZ and IS from rat whole brain membranes. Evaluation of the solvents was made on the basis

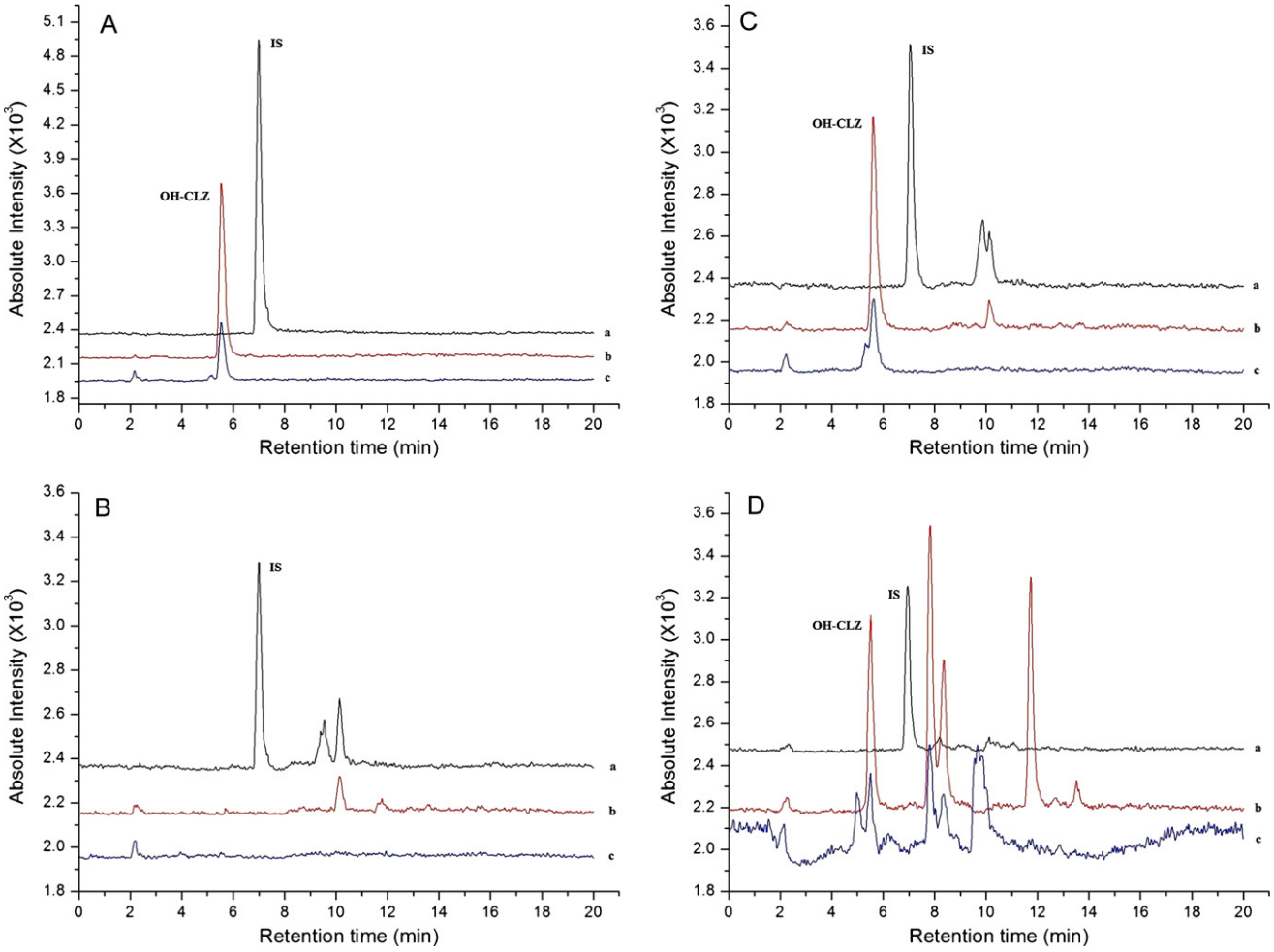

Fig. 3. Typical LC–MS chromatograms of standard OH-CLZ solution (A), blank rat whole brain membranes spiked with IS (B), blank rat whole brain membranes spiked with OH-CLZ and IS (C), CB region membranes of rat brain after incubation with 2.5 mM CLZ for 90 min (D). The concentration of OH-CLZ for panel A–C was 3.0 ng/mL, and the spiked IS concentration was 30.0 ng/mL.

of the peak areas of the OH-CLZ and IS from rat brain membranes. Fig. 5 summarizes the results obtained with the four organic solvents versus the peak areas. On the basis of these results, isopropyl ether was used as the extraction solvent for the following experiments.

3.3. Method validation

3.3.1. Calibration curve, linearity, limit of detection and limit of quantification

We have validated this LC–MS method to quantify OH-CLZ in membrane medium after a liquid–liquid extraction procedure. As shown in Fig. 3B, no peak was observed at the same retention time of OH-CLZ when blank rat brain membranes were tested after extraction, indicating that the liquid–liquid extraction method was selective.

To assess the linearity range of the proposed method, various concentrations of OH-CLZ in blank rat brain membranes ranging from 0.5 ng/mL to 20 ng/mL were extracted for analysis. The calibration curve was constructed by comparing the peak areas against the analyte concentrations. As shown in Table 1, a good linearity for OH-CLZ is obtained with correlation coefficient (R^2) value of 0.9976. The LOD and LOQ were determined with low concentration (0.05 and 0.16 ng/mL) and calculated with the S/Ns of 3 and 10 respectively.

3.3.2. Precision and accuracy

Intra-day precision was evaluated by analyses of QC samples (0.5, 3.0 and 20.0 ng/mL) at five replicates of each QC level on the same day. Inter-day precision was determined by repeated analyses of QC samples over 5 consecutive days. The concentration of each sample was determined using freshly prepared calibration standards. Assay precision was calculated using the relative standard deviation (RSD%). The accuracy (measured value/nominal value) is the degree of closeness of the determined value to the nominal true value under prescribed conditions. The intra-day and inter-day precision and accuracy of QC samples are summarized in Table 2. Intra-day precision ranged from 5.9 to 14.0% with an accuracy rang-

Table 1
Linearity characteristic of OH-CLZ analyzed by LC–MS method.

Compound	Concentration range (ng/mL)	Regression line			LOD (ng/mL)	LOQ (ng/mL)
		Slope	Intercept	R^2		
OH-CLZ	0.5–20	0.1925	0.0328	0.9976	0.05	0.16

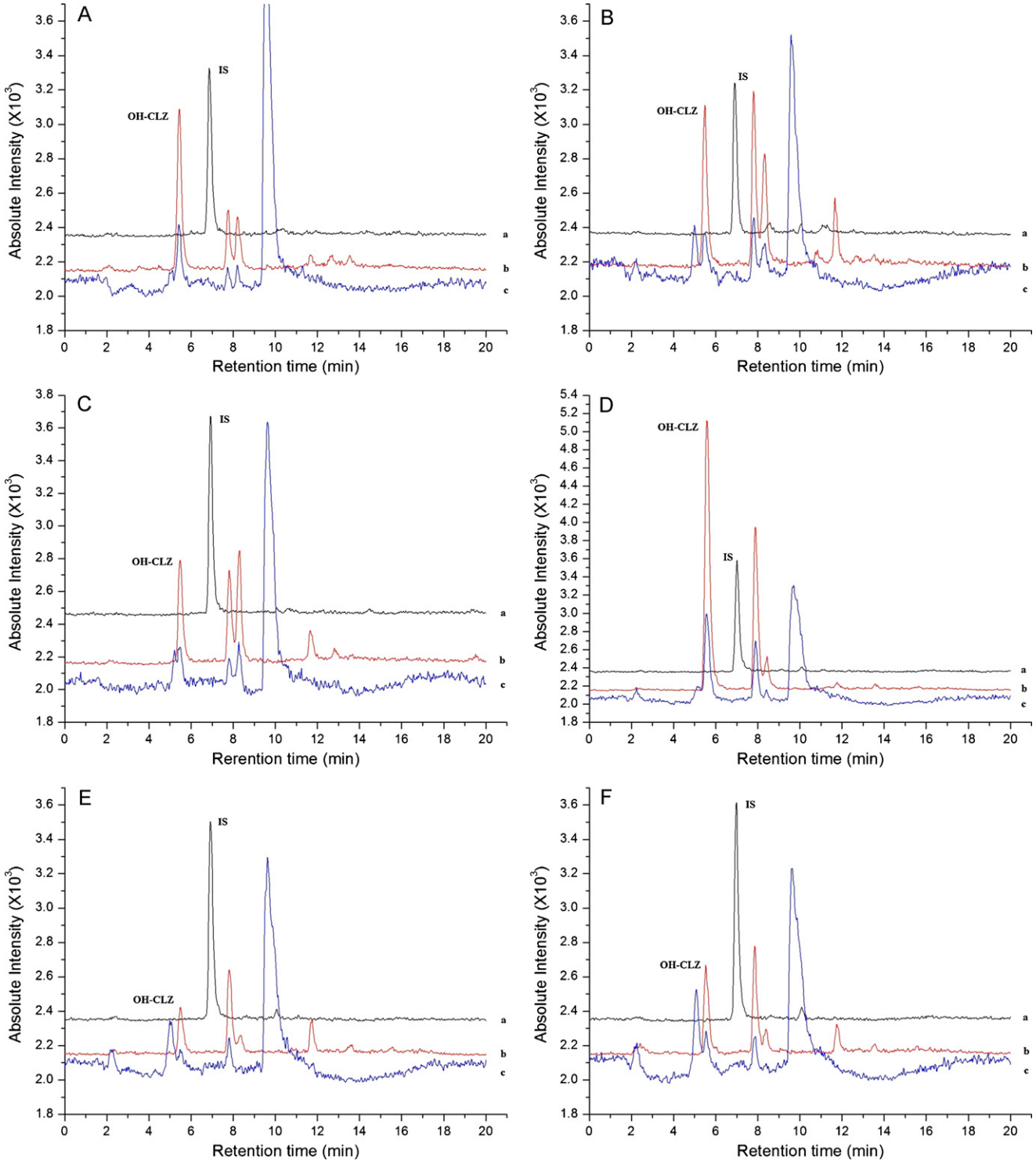

Fig. 4. Typical LC–MS chromatograms of (A) FC region, (B) HC region, (C) TH region, (D) OB region, (E) BS region, (F) ST region membranes of rat brain after incubation with 2.5 mM CLZ for 90 min. The spiked IS concentration was 30.0 ng/mL.

Table 2
Intra-day and inter-day repeatability and accuracy.

QC concentration (ng/mL)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Concentration found (ng/mL)	RSD (%)	Accuracy (%)	Concentration found (ng/mL)	RSD (%)	Accuracy (%)
0.5	0.46 ± 0.06	14.0	92.0	0.47 ± 0.07	14.9	94.0
3.0	2.8 ± 0.3	10.7	93.3	2.8 ± 0.4	14.3	93.3
20.0	20.2 ± 1.2	5.9	101.0	18.8 ± 1.1	6.0	94.0

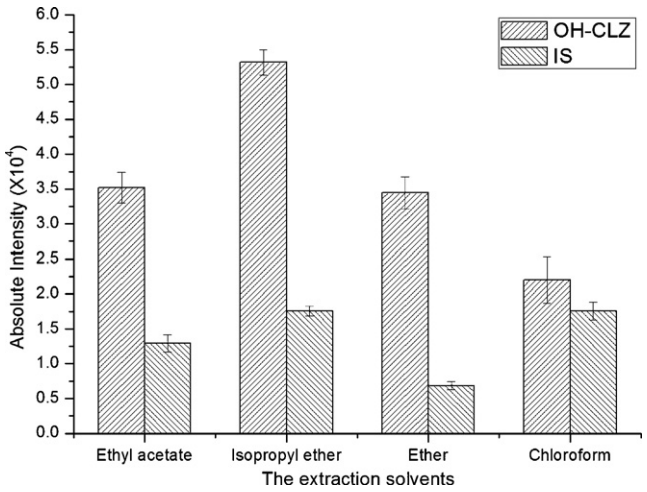

Fig. 5. Comparison of the extraction efficiencies of OH-CLZ and IS with different organic solvents. The spiked concentration of OH-CLZ and IS were 10.0 ng/mL and 30.0 ng/mL, respectively.

ing from 92.0 to 101.0%, and inter-day precision ranged from 6.0 to 14.9% with an accuracy from 93.3 to 94.0%. These data indicate that the present method is accurate, precise and reproducible for quantification of OH-CLZ in rat brain membranes with the OH-CLZ concentration from 0.5 ng/mL to 20.0 ng/mL.

3.3.3. Matrix effect and method recovery

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample [41]. The matrix effect and method recovery of this method was evaluated at three OH-CLZ concentration levels of 0.5 ng/mL, 3.0 ng/mL and 20.0 ng/mL, the IS concentration level was 30.0 ng/mL. Matrix effect was calculated by comparison of the mean peak areas of OH-CLZ spiked afterwards in blank whole brain membrane samples after the precipitation step and injected to those of OH-CLZ spiked in 20% acetonitrile and directly injected. If the matrix effect values exceed the range of 85–115%, an endogenous matrix effect is implied. As shown in Table 3, the results obtained were well within the acceptable limit, it indicated that there was no matrix effect of the analytes observed in this study. Meanwhile, method recovery was calculated by comparison of the mean peak areas of OH-CLZ extracted from whole brain membrane samples to those of injected standards. The extraction recovery of the assay, determined at three concentrations of 0.5, 3.0 and 20.0 ng/mL were 92.8, 87.1 and 86 .2%, respectively. Isopropyl ether chosen as the extraction solvent cannot only eliminate the interference of endogenous substances, but also meet the requirement of sensitivity for the assay.

Table 3
Matrix effect and method recovery of OH-CLZ and IS in whole brain membranes of rat.

	Concentration (ng/mL)		Matrix effect	Method recovery
OH-CLZ ($n=5$)	0.5	Mean (%)	103.2	92.8
		CV(%)	1.6	8.1
	3.0	Mean (%)	101.2	87.1
		CV(%)	4.4	10.0
	20.0	Mean (%)	95.5	86.2
		CV(%)	3.3	7.3
IS ($n=5$)	100.0	Mean (%)	90.1	90.3
		CV(%)	9.0	9.0

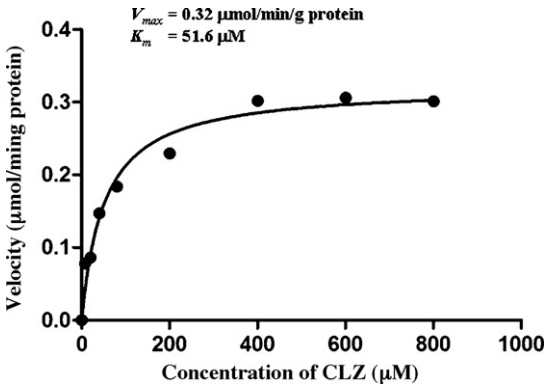

Fig. 6. Plot of OH-CLZ formation velocity following incubation of CLZ at the indicated concentrations (8–800 μM) with rat liver membranes at 37 °C for 90 min. The kinetic data were fit to a simple Michaelis–Menten equation.

3.4. Determination of the CYP2E1 enzyme activity in brain regions of rat

In order to demonstrate the reliability of the method to evaluate CYP2E1 activity in different rat brain regions, OH-CLZ which generated by incubation of CLZ with rat liver microsomes were also determined. Eight substrate concentrations ranging from 8 to 800 μM (8, 20, 40, 80, 200, 400, 600 and 800 μM) were performed to obtain kinetic parameters (K_m and V_{max}) of CLZ hydroxylation in rat liver microsomes. Fig. 6 showed the formation velocity of this reaction in microsomes of rat liver with our presented method. The estimated K_m and V_{max} values of rat liver microsomes incubated with CLZ were 51.6 μM and 320 pmol/mg protein/min, respectively. Our K_m and V_{max} values of rat liver microsomes are similar to those obtained previously by other researchers [24,27,42,43]. This demonstrated the developed method was reliable to evaluate CYP2E1 activity in different rat brain regions with the probe of CLZ. The kinetic parameters of K_m and V_{max} were estimated by analyzing Michaelis–Menten plots using Prism v4.0 software (GraphPad Software, San Diego, CA).

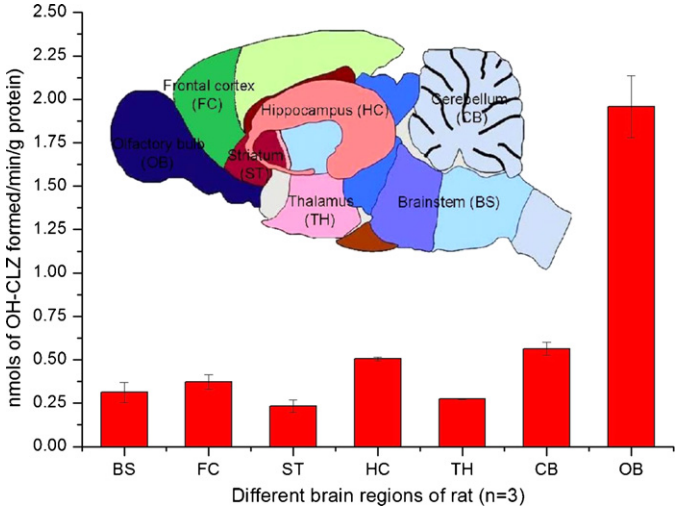

Fig. 7. CYP2E1 activity in different brain regions of rats. Membranes were prepared from brain regions of each rat including frontal cortex (FC), cerebellum (CB), brain stem (BS), hippocampus (HC), striatum (ST), thalamus (TH), and olfactory bulb (OB). The CYP2E1 activity was measured by incubating membrane protein (0.5 mg) with CLZ (0.25 mM) for 90 min and assaying the amount of formed OH-CLZ by the developed LC–MS method. Values are means ± SD ($n=3$, each brain region membranes were prepared from one rat).

This assay methodology was then applied to the experiment in which CLZ was incubated with membranes of different rat brain regions and the production of OH-CLZ measured, as an estimate of CYP2E1 activity. Firstly, we determined the protein concentrations, incubation times and substrate concentration which effected the formation of OH-CLZ. The hydroxylase activity in rat brain CB region membranes increased over a protein concentration of 0.25–1.5 mg incubation. The formation of OH-CLZ also increased over a time period of 30–180 min, and over a substrate concentration of 0.0025–2.5 mM (data not shown here).

Operating under linear conditions, the formation of OH-CLZ was readily detectable at substrate concentrations of 0.25 mM and 0.5 mg protein for incubation 90 min. We then examined CLZ 6-hydroxylase activity in different regions of each rat brain. Regional variation was observed in the activity of CLZ 6-hydroxylase in different brain regions of each healthy rat. The enzyme activity in the olfactory bulb and cerebellum were significantly higher than that in the cortex, hippocampus, striatum, thalamus, and brain stem regions (Fig. 7). Our results are consistent with those reported previously by other researchers [42,44], in which both the cerebellum and the olfactory bulb are known to have higher CYP2E1 content compared to other regions of the rat brain.

4. Conclusion

The study described herein demonstrates a simple, sensitive and reliable LC–MS assay for OH-CLZ in brain membranes from different regions with the substrate of CLZ. This method was applied to these incubations to measure the formation of OH-CLZ from CLZ as a probe for CYP2E1 activity. In previous studies, pooled brains from several rats were used in other reported literatures [44,45]. In present study, a sensitive method has been established to detect CYP2E1 activity with the probe of CLZ in different brain regions from single rat, which provides the possibility to future analyze the relationship between protein expression and catalytic activity of brain CYP2E1. Furthermore, this method shows an increased sensitivity of about 100-fold with the HPLC–UV method reported by Leclercq and Sherry [24,25], and about 10-fold increase with the HPLC–MS/MS method reported by Zhong and Tolonen [33,34]. Its high sensitivity makes this method attractive for the analysis of CYP2E1 activity in small brain regions of rat and low levels of CYP2E1 samples with the probe of chlorzoxazone.

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