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Development and validation of an UPLC-MS/MS method for the quantification of ethoxzolamide in plasma and bioequivalent buffers: Applications to absorption, brain distribution, and pharmacokinetic studies

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Abstract

The purpose of this study is to develop and validate an UPLC-MS/MS method to quantify ethoxzolamide in plasma (EZ) and apply the method to absorption, brain distribution, as well as pharmacokinetic studies. A C_{18} column was used with 0.1% of formic acid in acetonitrile and 0.1% of formic acid in water as the mobile phases to resolve EZ. The mass analysis was performed in a triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) with positive scan mode. The results show that the linear range of EZ is 4.88–10,000.00 nM. The intra-day variance is less than 12.43 % and the accuracy is between 88.88–08.00 %. The inter-day variance is less than 12.87 % and accuracy is between 89.27–115.89 %. Protein precipitation was performed using methanol to extract EZ from plasma and brain tissues. Only 40 μL of plasma is needed for analysis due to the high sensitivity of this method, which could be completed in less than three minutes. This method was used to study the pharmacokinetics of EZ in SD rats, and the transport of EZ in Caco-2 and MDCK-MDR1 overexpressing cell culture models. Our data show that EZ is not a substrate for p-glycoprotein (P-gp) and its entry into the brain may not limited by the blood-brain barrier.

Keywords

ethoxzolamide; UPLC-MS/MS; Pharmacokinetics; absorption; CNS distribution

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1. Introduction

Ethoxzolamide (EZ, Fig. 1), also known as ethoxyzolamide, is a sulfonamide medication used for the treatment of glaucoma, duodenal ulcers, epilepsy, and also used as a diuretic [1–3]. In addition, these classes of agents have potential as novel anti-obesity, anticancer, and anti-Alzheimer's Disease drugs [4, 5]. Pharmacological studies in experimental models of glaucoma have shown that EZ inhibits carbonic anhydrase (CAs) activity in eyes, decreases aqueous humor levels and results in the reduction of intraocular pressure [3]. It has also been reported that EZ decreases CA activity in the CNS and increases the threshold for seizure activity, suggesting a use for EZ in the treatment of epilepsy. In addition, EZ acts as a diuretic and can decrease the reabsorption of water, sodium, potassium, and bicarbonate [3].

As an inhibitor of CAs, EZ holds the promise as a potential candidate drug for the treatment of multiple diseases. It is well recognized that CAs are a group of zinc-containing metalloenzymes that catalyze reversible hydration of carbon dioxide to the bicarbonate ion. CAs are involved in numerous physiological and pathological processes, including gluconeogenesis, lipogenesis, ureagenesis, tumorigenicity, and the growth and virulence of various pathogens [4, 5]. EZ has been subjected to a few pre-clinical and clinical studies [3, 6]. However, only one analytical method, published 30 years ago, has been reported for the quantification of EZ in plasma and tissue samples from rabbit. In that method, ethyl acetate was used as extraction solvent, and 50% methanol in 1% acetic acid was used as isocratic elution. The linear range was from 0.03–0.5 microgram/mL. More importantly, this HPLC-based method had a sensitivity of only 0.03 microgram/mL (0.11 μM) [7], which is above the Kd (dissociation constant) of EZ (typically in the nM range) for CAs [8, 9]. Thus, there is a need for a more sensitive method to quantify EZ in biological samples.

Liquid chromatography hooked with mass spectrometry (LC–MS/MS) plays an important role in drug analysis especially from biological samples due to its inherent specificity, sensitivity, and speed. Usually an LC-MS method is more sensitive than a method with UV detector. LC-MS is generally accepted as the preferred technique for quantitating drugs and its metabolites in biological matrices (plasma, blood, serum, urine, and tissue). However, many factors, such as sample matrice, extraction solvent, may affect the ionization in mass spectrometer, which consequently affect the sensitivity. Therefore, a LC-MS method needs to be optimized before its application. In addition, validation is necessarily performed to verify that the data generated by this method is reliable.

In this study, we developed a sensitive and reproducible UPLC-MS/MS method for quantification of EZ in plasma, brain tissue, and bioequivalent buffers (e.g., HBSS). The method was successfully used to investigate the role of the p-glycoprotein (P-gp) transporter in the absorption of EZ using Caco-2 and MDCK-MDR1 overexpressing cell culture models. The distribution of EZ in the brains of SD rats after intravenous administration was determined using this method.

2. Experimental

2.1. Chemicals and reagents

Ethoxzolamide, sulpiride, and Hanks' balanced salt solution (powder form) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, and water (LC-MS grade) were purchased from EMD (Gibbstown NJ). All other materials (typically analytical grade) were used as received.

2.2. Instruments and conditions

2.2.1 UPLC system—was Waters Acquity™ UPLC system with a TUV detector. The column was Acquity UPLC BEH C_{18} column (50 × 2.1 mm I.D. 1.7 μM, Waters, Milford, MA, USA). The 0.1% formic acid was used as mobile phase A and acetonitrile was used as mobile phase B. The gradients was 0 to 0.5 min, 5 to 10 % B, 0.5 to 1.5 min, 10 to 95% B, 1.5 to 2.0 min, 95 % B, 2.0 to 2.5 min, 95 to 10 % B, 2.5 to 3.0 min, 10% B; flow rate was 0.55 ml/min, and the column temperature was 60°C.

2.2.2 MS system—MS analysis was performed using an API 5500 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/ MDS SCIEX, Foster City, CA, USA) equipped with a TurboIonSpray™. The concentration of EZ was determined by using the multiple reactions monitoring (MRM) scan type with ion pair transition to monitor the analyte. Unit mass resolution was set in both mass-resolving quadruple Q1 and Q3 in positive scan mode. The ionspray voltage was 5.5 kV and the ion source temperature was 400°C nebulizer gas (gas 1), nitrogen, 40 psi; turbo gas (gas 2), nitrogen 35 psi; curtain gas, nitrogen 30 psi.

2.3. Method validation

2.3.1. Calibration curve and LLOD—Calibration standards were prepared in 50 % methanol by diluting a stock solution of EZ to final concentrations of 10,000.00 5,000.00, 2,500.00, 1,250.00, 625.00, 312.50, 156.00, 78.00, 39.00, 19.50, 9.75, 4.88, 2.44, 1.22, and 0.61 nM respectively. To prepare standard curve in buffer, the stock solution was diluted into HBSS buffer directly and 20 μL of I.S was added. To prepare standard curve in plasma, blank plasma (40 μL) was mixed with 40 μL of standard curve samples prepared in 50% methanol and 160 μL of I.S. After centrifugation at 20,000 g for 15 min, the supernatant solution was transferred to a new tube and dried under a stream of nitrogen. The residue was reconstituted in 80 μL of 50% methanol and centrifuged at 20,000 g for 15 min for injection. To prepare standard curve in brain tissue, a mixture of 40 μL of standard curve samples prepared in 50% methanol with blank tissues (about 50 mg) were homogenized in I.S. (1.0 ml) and centrifuged at 20,000 g for 15 min. The supernatant (0.8 ml) was collected, dried under N2, and resuspended as described above. The linearity of each calibration curve was determined by plotting the ratio of the peak areas of EZ to internal standard (I.S.; sulpiride in methanol) in rat plasma and homogenized brains. A leastsquares linear regression method ($1/\times^2$ weight) was used to determine the slope, intercept and correlation coefficient of linear regression equation. The lower limit of detection (LLOD) was defined based on a signal-to-noise ratio of 10:1.

2.3.2. Precision and accuracy—The "intra-day" and "inter-day" precision and accuracy of the method were determined with quality control (QC) samples at three different concentrations (six injections for each concentration) on the same day or on three different days [10].

2.3.3. Extraction recovery and matrix effect—The extraction recoveries of EZ was determined by comparing the relative peak areas obtained from blank plasma or blank homogenized brain extracts spiked with analytes, and those obtained from water spiked with the same amount of analytes. Matrix effect was determined by comparing the peak areas of blank plasma or homogenized brain extracts spiked with analytes and I.S. with those of the standard solutions dried and reconstituted with a mobile phase. These evaluations were performed according to the recommended validation procedures reported by Matuszewski [11].

2.3.4. Stability—The stability of EZ in rat plasma was determined by analyzing three replicates of QC samples at three different concentrations following storage at 25 °C for 4 h, at −80 °C for 7 days, and after going through three freeze-thaw cycles (−80 °C and 25 °C). The samples were prepared by diluting the stock solution in 50% methanol then adding this solution to plasma to achieve final concentrations of 5000.00, 312.50, and 9.75 nM (20% of solvent in plasma). Stability was expressed as the ratio of the mean peak area (triplicate samples) of an analyte at different time points to the mean peak area of the same analyte at time zero multiplied by 100.

2.4. Pharmacokinetics study

2.4.1. Animals—Sprague Dawley rats (male, 300–325 g) were obtained from Harlan Laboratory (Indianapolis, IN) and housed in an environmentally controlled room (temperature: 25 ± 2 °C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week prior to experimental manipulation. The rats were given *ad libitum* food and water. The animal protocols used in this study were approved by the University of Texas's Institutional Animal Care and Use Committee.

2.4.2. Pharmacokinetics and brain distribution experimental design—The animals were randomly selected into 2 groups (n=6 each group) and EZ was administered at a dose of 0.18 mg/kg (in PEG 300: ethanol, 1:1) via i.v. injection through the tail vein. Blood samples (about 50–100 μL) were collected in heparinized tubes at 0, 15, 30, 60, 120, 180, 240, 360, 540, and 1440 min post-injection, via tail snip with isoflurane as anesthetic. Plasma samples were prepared and stored at −80 °C until analysis. To study the distribution in brain, rats in group 1 were scarified at 6 hours and rats in group 2 were scarified at 24 hours to collect the brain tissues. Those blood samples from group 2 were analyzed to generated PK profile.

2.4.3. Sample preparation for UPLC—Plasma samples (40 μL) were mixed with 40 μL of 50% methanol and 160 μL of I.S. The mixture was vortexed for 1 min. After centrifugation at 20,000 g for 15 min, the supernatant solution was transferred to a new tube and dried under a stream of nitrogen. The residue was reconstituted in 80 μL of 50%

methanol and centrifuged at 15,000 rpm for 15 min. For determining EZ levels in the brain, animals were transcardially perfused with ice-cold saline, then hippocampal and cortical tissues removed and immediately frozen. Tissues were homogenized in 40 μL of 50% methanol and I.S. (1.0 ml) and centrifuged at 15,000 rpm. The supernatant (0.8 ml) was collected, dried under N_2, and resuspended as described above. 10 μL of supernatant was injected into the UPLC–MS/MS system for analysis. The density of the blood is treated as 1g/mL in the tissue distribution study.

2.4.4. Preparation of standard and quality control samples—Calibration standards were prepared as described in section 2.3.1. The quality control (QC) samples were prepared at three different concentrations essentially as described above for the calibration standards.

2.4.5. Pharmacokinetics parameter calculation—The pharmacokinetic parameters of EZ were calculated by the non-compartmental method, using the *Win-Nonlin 3.3* (Pharsight Corporation, Mountain View, California) program.

2.5 Transport experiments in the Caco-2, MDCK-MDR1 cell culture models

Cell cultures were prepared as described previously by our laboratory [12–14]. Cells were used between passages 41–49. Briefly, a cell monolayer was prepared by seeding 400,000 cells per insert (Nunc, surface area=4.2 cm^2, 3 μm pore size). Cells were maintained at 37 °C under 90% humidity and 5% CO_2. Monolayers were used between 19 and 22 days after seeding for Caco-2 cells and 4–5 days for MDCK-MDR1 cells. The integrity of each monolayer was checked by measuring the transepithelial electrical resistance (TEER; Millicell ERS) before the experiment. The normal TEER values obtained were above 500 Ω·cm^2 for Caco-2 cell and above 100 Ω·cm^2 for MDCK-MDR1 cells. HBSS (9.8 g/mL) supplemented with $NaHCO_3$ (0.37 g/L), HEPES (5.96 g/L), and glucose (3.5 g/L) was used for all experiments after the pH had been adjusted to 7.4.

The experimental protocol and calculations were described in our previous reports [12–14]. Briefly, 10 μM solution of EZ in HBSS buffer was loaded onto the apical or basolateral (donor) side. Five donor samples (500 μL) and five receiver samples (500 μL) were taken at 0, 1, 2, 3, and 4 h followed by the addition of 500 μL of fresh donor solution to the donor side or 500 μL or fresh buffer to the receiver side. The samples were then analyzed by UPLC-MS/MS. The apparent permeability coefficient (P) was determined by the equation

$$P = \left(\frac{dQ}{dt}\right) / (A \times C0)$$

where dQ/dt is the drug permeation rate (μmol/s), A is the surface area of the epithelium (cm^2), and C0 is the initial concentration in the donor compartment at time 0 (mM).

3. Results and discussion

3.1 Optimization of the UPLC-MS/MS condition

Different mobile and stationary phases were tested to enhance the sensitivity of detection of EZ. Methanol, acetonitrile, 2.5 mM ammonia acetate (pH = 7.6), 0.1–0.5 % formic acid, and 100% water were tested as potential mobile phases. Both C_{18} and C_8 columns were evaluated as stationary phases. Based on the intensity of the signal, and the shape of the peak, acetonitrile and 0.1% formic acid and C_{18} column were found to be the optimal mobile and stationary phases, respectively. In addition, maintaining the column temperature at 60°C and the flow rate at 0.55 ml/min yielded sharp and symmetrical peaks (Fig 2A).

For MS/MS, both positive and negative scan modes were evaluated. A typical MS/MS spectra is in figure 2B. Based on the intensity of the analytes, positive scan mode was found to be more sensitive. Multiple reaction monitoring (MRM) scan type was used to improve the specificity. The compound and instrument dependent parameters were optimized by tuning the analytes separately. The results are shown in Table 1.

3.2 Method validation

3.2.1 Linearity and lower limit of detection (LLOD)—Method validation was conducted using blank HBSS buffer, untreated rat plasma, and brain tissue samples. The standard curve was linear from 4.88nM to 10,000.00 nM ($R^2 > 0.99$) in plasma and brain tissue, and from 5,000 nM to 1.22nM in HBSS buffer respectively. The accuracy of each standard sample was in the acceptable range (87.31–112.52 %) [10, 15]. The lower limit of detection (LLOD) was determined to be 2.44 nM in plasma and tissue, and 0.6 nM in HBSS buffer. The accuracy of LLOQ samples in plasma, tissue, and buffer are 87.31%, 112.77% and 92.32% respectively.

3.2.2 Accuracy and precision—Accuracy, intra-day and inter-day variability were determined by running six replicates of QC samples at three different concentrations of EZ in rat plasma, brain tissue, and HBSS buffer. The accuracy and reproducibility of these measurements are shown in Table 2 and were found to be in the acceptable range (85–115%) according to FDA guidance [10, 15].

3.2.3 Recovery, matrix effect and stability—The mean extraction recoveries determined using three replicates of QC samples at three concentration levels (the same concentrations as QC sample) in rat plasma, brain tissue, or HBSS buffer are shown in Table 3. A one-step precipitation was used to extract EZ from the plasma samples, brain tissue, or HBSS buffer. The result showed the recoveries were higher than 80 % for EZ at all of the three concentrations tested.

To test the matrix effects that may impact UPLC-MS analysis, the relative peak areas of the analyte spiking into the evaporated plasma, brain tissue, or HBSS samples at three concentration levels were comparable to similarly prepared aqueous standard solutions. Three different concentrations of analyte were tested. As shown in Table 3, no measurable matrix effect was observed.

The stability of EZ in rat plasma was evaluated by analyzing three replicates of QC samples at three different concentrations (5000.00, 312.50, 9.75 nM) following storage at 25 °C for 4 h, at −20 °C for 8 h, at −80 °C for 7 days, and after going through three freeze-thaw cycles (from −80 °C to 25 °C). The recovery of EZ was found to be 85–115% in all the conditions tested.

3.5 Application of the method to study transport of EZ in cell culture models

The validated method was used to determine the transport of EZ in Caco-2 and MDCK-MDR1 cell culture models. In Caco-2 cells, the absorption permeability (from apical to basolateral side, A to B) of EZ was $1.59 \pm 0.15 \times 10^{-5}$ cm/sec (Fig 3). This rate of permeability may translate to more than 70% absorption in the human intestine following oral administration [16]. The permeability from basolateral to apical side of EZ was $1.49 \pm 0.49 \times 10^{-5}$ cm/sec, a value similar to that of absorption permeability, suggesting that EZ follows the passive diffusion mechanism (Fig 3).

P-gp is the major efflux transporter that is expressed at high levels on the luminal surface of brain endothelial cells. As this transporter can limit drug absorption into the brain, we investigated the role of P-gp in the absorption of EZ in MDCK-MDR1 cells over-expressing P-gp. The permeability of EZ in non-overexpressing MDCK cells was determined to be $1.39 \pm 0.15 \times 10^{-5}$ cm/sec, and P-gp overexpressing cells were found to have a permeability to EZ that was not significantly different from non-overexpressing cells ($p > 0.05$, $1.83 \pm 0.73 \times 10^{-5}$ cm/sec; Fig 3), suggesting that EZ is transported by these cells in a P-gp independent manner.

3.6 Application of the method to determine the pharmacokinetics and brain distribution of EZ in rats after i.v. administration

The validated method was employed to study serum levels and brain distribution of EZ in Sprague Dawley rats. Mean plasma concentration as a function of time after *i.v.* (0.18 mg/kg dose) administration of EZ is shown in Fig 4. The C*max* of i.v. administered EZ was determined to be 8.75 μM, the T*max* was 26.30 minutes, and the $AUC_{0-\infty}$ was 5098.89 min*μM (Table 4). Tmax in this experiment was 26.30 min with i.v. administration suggested that the drug may has dissolution issue in the blood. More investigations are needed to explain this observation. Measurement of EZ in brain extracts showed that the amounts of EZ in the hippocampus were 1.47 ± 0.52 and 3.13 ± 0.54 nM/gram of tissue at 6 and 24 hours, respectively. In the cortex, the amounts were found to be 0.45 ± 0.25 and 1.87 ± 1.96 nM/gram of tissue at 6 and 24 hours (Fig 5). Interestingly, we observed that the plasma concentration of EZ at the 6hr time point was higher than that detected in either the hippocampus or cortex. In contrast, the plasma concentration of EZ at 24hr was significantly lower that the concentrations recorded in the hippocampus and cortex at this time point (Fig 5). These CNS concentrations, especially in the hippocampus, are within the range of concentration necessary to inhibit carbonic anhydrase. The ratio of brain/plasma concentration at 24 hr post administration was significantly larger than 1, suggesting that EZ have a higher concentration in the brain.

4. Conclusion

A rapid, sensitive, and specific UPLC-MS/MS method has been developed and validated for the quantification of ethoxzolamide in plasma, brain tissue, and HBSS buffers. The main advantages of this method are: 1) high sensitive (LLOQ, 4.8 nM); 2) rapid analysis (3.0 min); 3) simple sample preparation procedure and good recovery and minor matrix effect. Application of this analysis method to i.v. pharmacokinetic and brain distribution studies showed that in rats. In addition, the distribution of EZ in the brain may not be limited by the blood-brain barrier. This method would be of valuable for human clinical studies because of its high sensitivity and requirement for small sample volumes.

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Abbreviations

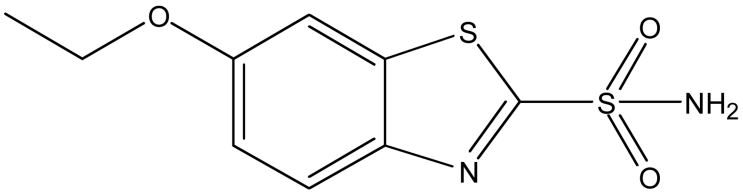
UPLC	ultra-performance liquid chromatography
I.S.	internal standard
DP	declustering potential
CE	collision energy
CXP	collision cell exit potential
AUC	area under the curve
QC	quality control
LLOQ	lower limit of quantification
EZ	Ethoxzolamide
CA	carbonic anhydrase
CNS	Central nervous system
MPA	mobile phase A
MPB	mobile phase B

References

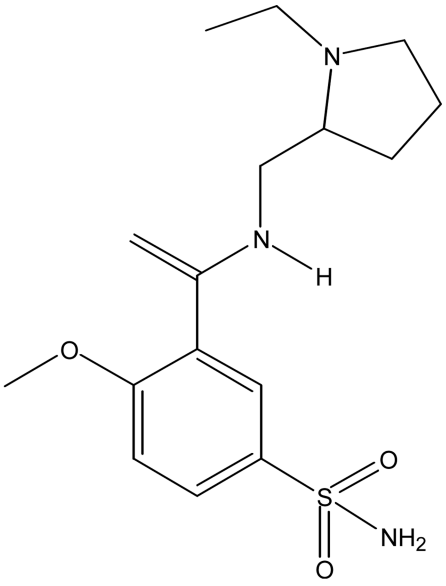
1. Puscas I, Buzas G. Int J Clin Pharmacol Ther Toxicol. 1986; 24:97–99. [PubMed: 3957499]
2. Barbarino F, Toganel E, Brilinschi C. Ann N Y Acad Sci. 1984; 429:601–603. [PubMed: 6234837]
3. Maren TH, Brechue WF, Bar-Ilan A. Exp Eye Res. 1992; 55:73–79. [PubMed: 1397133]
4. Supuran CT. Nat Rev Drug Discov. 2008; 7:168–181. [PubMed: 18167490]
5. N FM, Pura M, A SBW, et al. Eur J Endocrinol. 2008; 158:705–709. [PubMed: 18426830]
6. Shrader CE, Thomas JV, Simmons RJ. Am J Ophthalmol. 1983; 96:730–733. [PubMed: 6660261]
7. Eller MG, Schoenwald RD. J Pharm Sci. 1984; 73:1261–1264. [PubMed: 6491947]
8. Mack ET, Snyder PW, Perez-Castillejos R, et al. J Am Chem Soc. 2012; 134:333–345. [PubMed: 22088143]
9. Baranauskiene L, Matulis D. BMC Biophys. 2012; 5:12. [PubMed: 22676044]

10. Ramalingam P, Ko YT. J Chromatogr B Analyt Technol Biomed Life Sci. 2014; 969:101–108.
11. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Anal Chem. 2003; 75:3019–3030. [PubMed: 12964746]
12. Yang Z, Gao S, Yin T, et al. J Pharm Biomed Anal. 2010; 51:1120–1127. [PubMed: 20034755]
13. Gao S, Yang Z, Yin T, et al. J Pharm Biomed Anal. 2011; 55:288–293. [PubMed: 21349678]
14. Yang Z, Gao S, Wang J, et al. Drug Metab Dispos. 2011; 39:1866–1872. [PubMed: 21757611]
15. Xu RN, Fan L, Rieser MJ, et al. J Pharm Biomed Anal. 2007; 44:342–355. [PubMed: 17360141]
16. Artursson P, Karlsson J. Biochem Biophys Res Commun. 1991; 175:880–885. [PubMed: 1673839]

1. An UPLC-MS/MS method to quantify ethoxzolamide was developed and validated.
2. The sensitive and robust method was used in absorption study in cell culture model.
3. The sensitive and robust method was used in pharmacokinetic study in SD rats.
4. The sensitive and robust method was used in brain distribution study in SD rats.



Ethoxzolamide (EZ)



Sulpiride (I.S.)

Figure 1.
Chemical structures of EZ and Sulpride (used as I.S.)

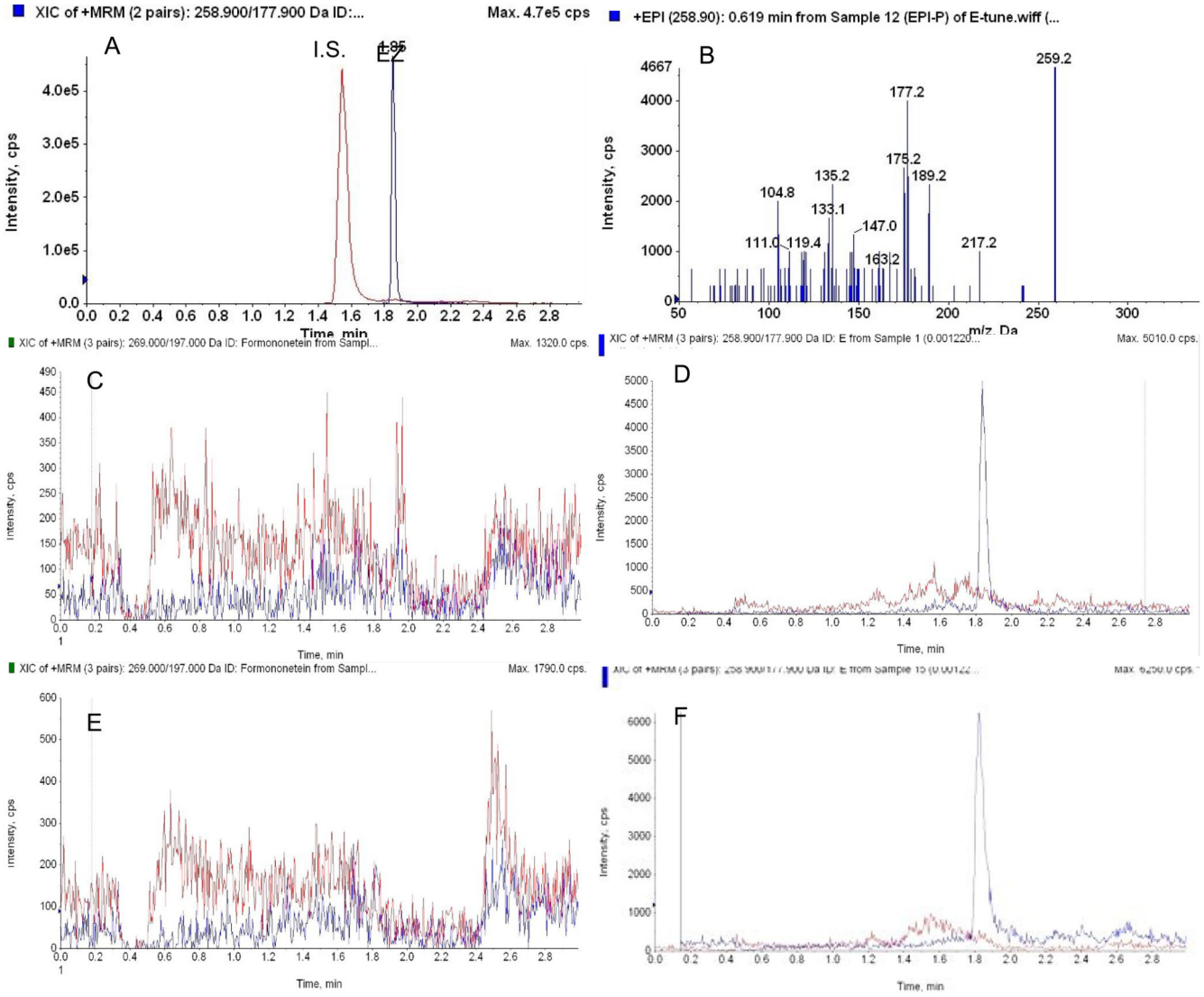


Figure 2.

Representative MRM chromatograms of EZ and I.S. in rat plasma (A) and the MS/MS spectrum of EZ (B). C and D is blank plasma (C) or blank plasma spiked with EZ (4.88 nM, D) respectively, and E and F is blank brain homogenate (E) and blank brain homogenate spiked with EZ (4.88 nM, F).

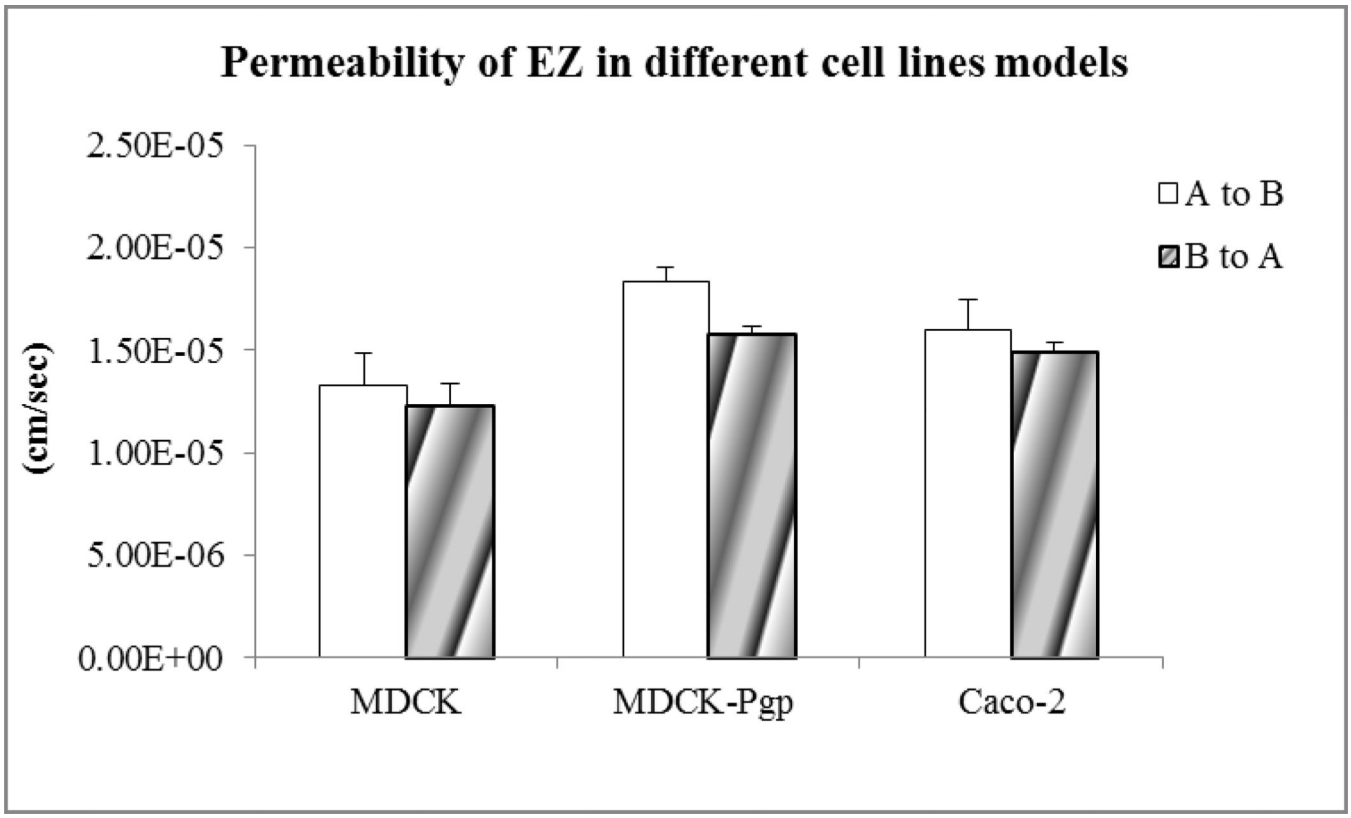


Figure 3.

Bidirectional transport of EZ across Caco-2 and P-gp overexpressing MDCK-MDR1 cell monolayers. The buffer used in both donor and receiver sides was HBSS (pH = 7.4). The donor side concentrations of EZ (both apical and basal sides were tested) were always 10 μM. Experiments were performed at 37°C. Each data point represents the average of three replications. Error bars indicate the standard deviation.

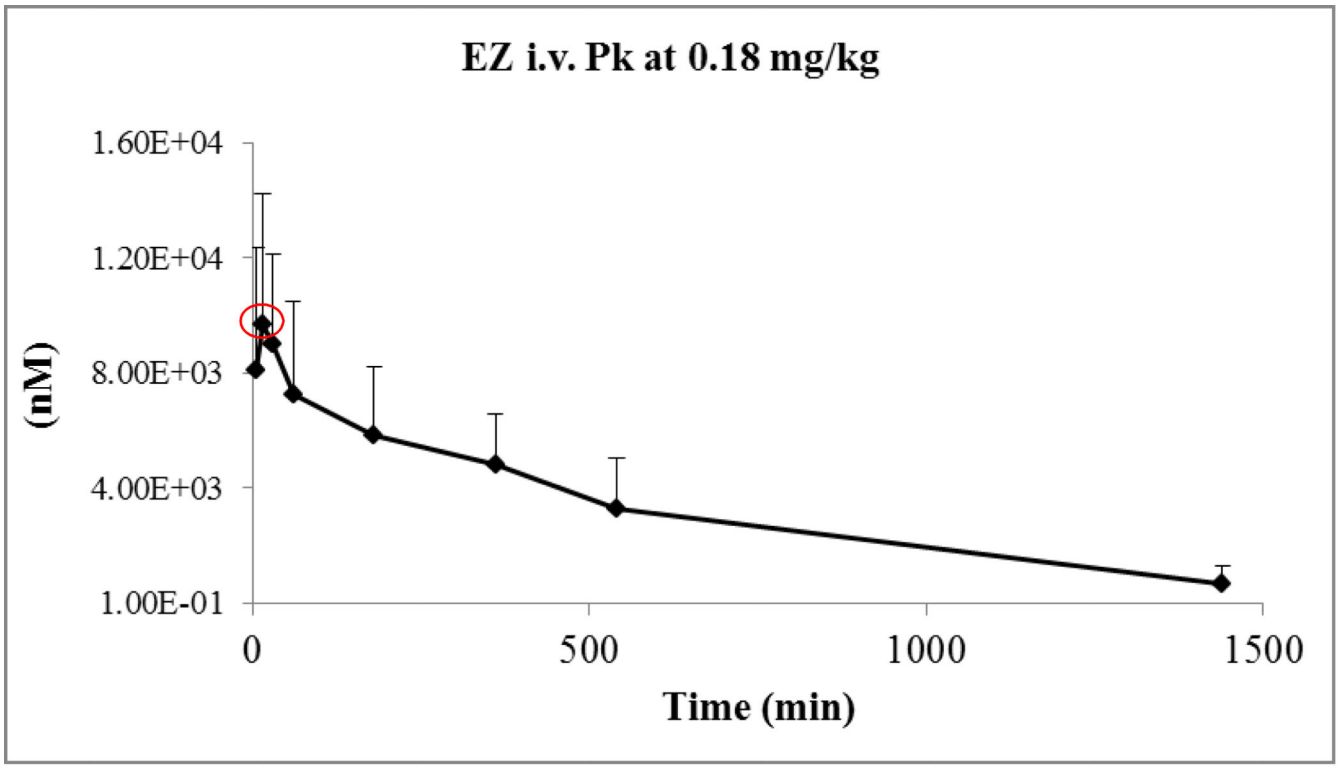


Figure 4.

Plasma concentrations of EZ after *i.v*. administration of 0.18 mg/kg in SD rats (n=6). Plasma samples (40 μL) were mixed with 50 % methanol (40 μL), and spiked with I.S. (160 μL, sulpride in methanol, 0.2 μM). The concentration of EZ was determined in six replicates. Error bars indicate standard deviations. The circled concentration was out of the linear range.

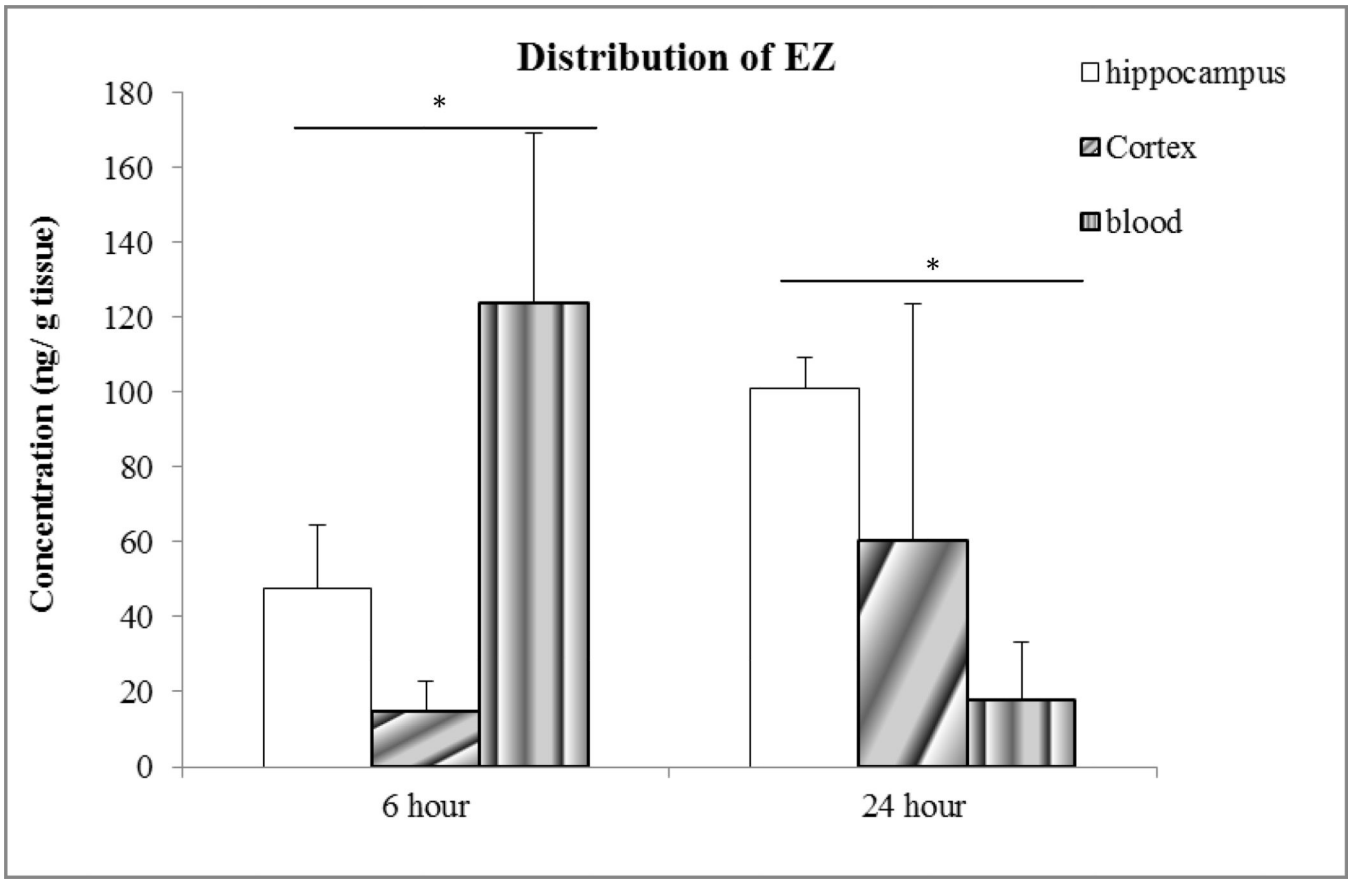


Figure 5.

Hippocampal and cortical distribution of EZ (n=6). EZ was administrated i.v. at 0.18mg/kg. Hippocampal and cortical tissues were harvested 6 hours and 24 hours after injection. Each data point is the average of six determinations. Error bars indicate standard deviation.

Table 1

Compound-dependent parameters in UPLC-MS analysis

Compound	Q1(*m/z*)	Q3(*m/z*)	Dwell time (ms)	DP (V)	EP(V)	CE(V)	CXP(V)
Ethoxzolamide (EZ)	258.9	177.2	100	161	10	23	12
Sulpiride (I.S.)	342.0	112.0	100	96	10	32	9

Table 2

Calibration curve, precession, recovery, and matrix effect of EZ.

	Linear range (nM)	Concentration (nM)	Intra-day		Inter-day		Recovery		Matrix Effect	
			Accuracy (bias, %)	Precession (CV %)	Accuracy (bias, %)	Precession (CV %)	Mean (%)	SD	Mean (%)	SD
Plasma	10,000.00-4.88	9.75	102.80	5.06	119.35	12.87	97.63	7.80	87.04	11.67
		312.5	101.20	4.97	115.89	12.50	82.71	3.37	85.50	8.59
		5,000.00	106.65	6.20	114.73	11.05	80.50	13.95	100.70	7.63
Brain tissue	10,000.00-4.88	9.75	91.19	12.43	89.27	4.83	98.58	12.17	90.47	13.97
		312.5	108.00	7.46	104.45	2.66	105.22	13.59	98.13	12.00
		5,000.00	106.22	2.14	107.11	4.30	106.23	5.74	100.44	5.59
HBSS buffer	5,000.00-1.22	4.88	106.44	4.05	89.02	3.07	91.35	6.00	97.73	3.53
		312.5	88.88	1.89	92.13	2.20	98.11	3.23	99.93	2.12
		5,000.00	100.5	1.84	107.34	3.77	98.83	4.96	101.97	5.57

Table 3

Pharmacokinetics parameters of EZ after *i.v.* administration at 0.18 mg/kg dose in SD rats (n = 6).

Tmax **(min)**	***Cmax*** **(μM)**	$AUC_{0-\infty}$ **(min*μM)**	$t_{1/2}$ **(min)**	**Cl_F_pred (mg/(min* μM)/kg)**
26.30 ± 14.78	8.75 ± 3.9	5098.89 ± 1524.12	433.86 ± 160.09	$3.78 \times 10^{-5} \pm 1.0510^{-5}$