Development and Validation of a Simple and Reliable LC-MS/MS Method for the Determination of Acetazolamide, an Effective Carbonic Anhydrase Inhibitor, in Plasma and its Application to a Pharmacokinetic Study

Drug Res 2014; 64: 499–504

For personal use only.
No commercial use, no depositing in repositories.
Development and Validation of a Simple and Reliable LC-MS/MS Method for the Determination of Acetazolamide, an Effective Carbonic Anhydrase Inhibitor, in Plasma and its Application to a Pharmacokinetic Study

Authors
X. Li, N. Li, C. Wang, S. Deng, X. Sun, W. Zhang, W. Gao, D. Zhao, Y. Lu, X. Chen

Affiliations
1 Center of Drug Metabolism and Pharmacokinetics, Faculty of Pharmacy, China Pharmaceutical University, Nanjing, China
2 Department of Membrane Transport and Biopharmaceutics, Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan

Key words
acetazolamide
LC-MS/MS
pharmacokinetics
beagle dogs

Abstract
A simple, rapid and accurate liquid chromatography – tandem mass spectrometry method was developed and validated for the quantification of acetazolamide in beagle plasma using sulfadiazine as the internal standard. After extraction by acetonitrile, the analytes were separated by a rapid gradient elution with acetonitrile and water as the mobile phase on a SHIMADZU VP-ODS C18 column and then detected by an API 4000 triple-quadrupole mass spectrometer using electrospray ionization in negative ion mode.

Introduction
Acetazolamide, N-(5-sulamoyl-1, 3, 4-thiadiazol-2-yl) acetamide, is an effective carbonic anhydrase inhibitor mainly used for the treatment of glaucoma at its early stage. Carbonic anhydrase can reversibly catalyze the hydration of CO₂ and dehydration of carbonic acid. The reaction produces HCO₃⁻ ion which is responsible for the transportation of Na⁺ ion into the eye. Then the aqueous humor that can cause the rise of intraocular pressure (IOP) is formed by water and Na⁺ ion inside the eye. And acetazolamide can lower the level of HCO₃⁻ ion by inhibiting carbonic anhydrase thereby reducing IOP [1]. Thus, acetazolamide can be effectively applied to the treatment of various types of glaucoma.

Recently, new applications of acetazolamide have been discovered and studied. For instance, it can be used for the preventive treatment of acute and chronic mountain sickness [2–4]. And it may be a potential target for controlling pancreatic cancer by regulating biological behavior of pancreatic cancer cells through inhibition of carbonic anhydrase (CA) I and II [5]. It can also effectively cure refractory epilepsies [6] and cerebrovascular pathema [7], etc. Thus it can be seen that the application of acetazolamide is very extensive and important.

While reports about the side effects of acetazolamide including acute kidney disease, hypokalemia and dermatitis medicamentosus have increased gradually in recent years, which has limited its application. On considering these problems, acetazolamide extended-release capsule (AEC) has been developed. Compared with conventional formulations, its dosing interval was significantly prolonged and plasma concentration was more stable and consistent. Thus, the compliance of patients and safety of the drug could be well improved.

Since AEC has such apparent advantages over conventional formulations, knowing its pharmacokinetic behavior is of great significance for guiding its clinical use. Therefore, practical and effective analytical methods for the determination of plasma acetazolamide and evaluation of its sustained release effect are urgently demanded.

Earlier methods including liquid chromatography-ultraviolet detection (LC-UV) for the analysis of acetazolamide in biological fluids have been described in some publications [8–11], but these methods were not applicable for pharmacokinetic studies owing to their limited selectivity and com-
plex sample preparation like PH dependent solvent extraction, etc. More recently, LC-mass spectrometry (LC-MS) [12,13] and LC-tandem mass spectrometric (LC-MS/MS) method [14–20] have been established. But instead of analyzing plasma concentration of acetazolamide, most of these methods were applied for the determination of acetazolamide in abiotic samples or for screening diuretics in human urine, and the whole analytical period was too long. Even with high selectivity, the sample preparation processes were also very time-consuming and large quantity of biological samples was needed. To sum up, none of these methods can be perfectly suitable for the present high-throughput pharmacokinetic study of acetazolamide in beagle dogs. Therefore, the main goal of this study was to establish and validate a simple, practical and accurate LC-MS/MS method with simplified sample preparation procedures to determine plasma acetazolamide, and further apply it to the pharmacokinetic study of acetazolamide after oral administration of 500 mg AEC to beagle dogs.

Materials and Methods

Chemicals and reagents

The reference substances of acetazolamide (purity > 99.7%, Fig. 1a) and the internal standard (IS) sulfadiazine (purity > 99.9%, Fig. 1b) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Test formulation of AEC was developed and provided by Henan Zhongshuai Medical Technology Development Co, Ltd. (Zhengzhou, China). Reference formulation of AEC (trade name: DIAMOX® SEQUELS®) was developed by Duramed Pharmaceuticals, Inc. subsidiary of BarrPharmaceuticals, Inc. (Cincinnati, USA). HPLC-grade methanol and acetonitrile were purchased from Tedia Company Inc. (Fairfield, OH, USA). Formic acid and other chemicals of analytical grade were provided by Nanjing chemical reagent Co., Ltd (Nanjing, China). Water was distilled and purified using a Milli-Q System (Millipore, MA, USA).

Instrumentation and LC-MS/MS conditions

The LC-MS/MS system consisted of a Shimadzu LC-10ADvp series HPLC instrument (Kyoto, Japan) equipped with a PerkinElmer series 200 autosampler (Massachusetts, USA), an API 4000 triple-quadrupole mass spectrometer equipped with an electrospray ionization source (Applied Biosystems Sciex, Toronto, Canada). Applied Biosystems Analyst software version 1.5.1 package was used to control the LC-MS/MS system for analytical data acquisition and processing. Acetazolamide and IS were separated on a Shimadzu VP-ODS C18 column (150 mm × 2.0 mm, i.d. 5 μm) (Shimadzu, Kyoto, Japan) at a column temperature of 35 °C. The mobile phase containing A (distilled deionized water) and B (acetonitrile) was pumped at a flow rate of 0.35 mL/min. The gradient program was as follows: 0 min 10 % B, 1.0 min 10 % B, 1.5 min 60 % B, 3.5 min 60 % B, 4.0 min 10 % B, 5.5 min stop. The sample injection volume was 5 μL with a total run time of 5.5 min. The mass spectrometer was operated in negative ion mode with multiple reaction monitoring (MRM) at unit resolution for the detection of m/z 220.9 → 83.3 [M-H] – (acetazolamide) and m/z 248.9 → 185.0 [M-H] – (IS). Fig. 2 shows the full-scan negative product ion mass spectra of acetazolamide and sulfadiazine.

---

**Fig. 1** Chemical structures of acetazolamide a and sulfadiazine b.

**Fig. 2** Full-scan negative product ion mass spectra of analytes: a acetazolamide and b sulfadiazine.
zolamide and IS. The optimized working parameters for mass spectrometer were as follows: dwell time, 200 ms; ion spray voltage, \(-4.5\) kV; gas temperature, 400 °C; collision-activated dissociation (CAD) 4 psi; declustering potential (DP), \(-50\) V; nebulizer gas (N$_2$), 20 psi; curtain gas (N$_2$), 10 psi; auxiliary gas (N$_2$), 20 psi; collision energy, \(-28\) V (acetazolamide) and \(-27\) V (IS).

Preparation of stock and working solutions

The stock solutions of acetazolamide and IS were separately prepared in methanol at the concentration of 1 mg mL$^{-1}$ and stored in brown volumetric flasks at 4 °C before use. A series of working solutions of acetazolamide (0.4, 1, 2, 4, 10, 20, 40, 100 μg/mL) and IS standard solution (2 μg/mL) were prepared by diluting stock solutions of the analyte and IS with the mobile phase.

Calibration standards and quality control samples

The calibration standards of acetazolamide were prepared by spiking acetazolamide working solutions into equal volume of blank beagle plasma to obtain the series concentrations of 0.2, 0.5, 1, 2, 5, 10, 20, 50 μg/mL. Quality control (QC) samples were prepared in the same way as calibration standards to achieve the final concentrations of 0.5, 5 and 40 μg/mL. All samples were stored at 4 °C until analysis.

Plasma sample preparation

For the preparation of plasma samples, a 100 μL of aliquot plasma sample was mixed with 100 μL of IS standard solution (2 μg/mL). The plasma mixture was precipitated by 600 μL of acetonitrile, vortex-mixed for 1 min and then centrifuged at 16 000 × g for 10 min. Finally, 5 μL of the supernatant was directly injected onto the LC-MS/MS system for analysis.

Application to a pharmacokinetic study

6 healthy beagle dogs (3 males and 3 females, 9.6 ± 0.23 Kg) were purchased from Shanghai Xin’gang laboratory animal center (Shanghai, China). The whole process of the animal experiment was carried out according to the guidance of the Animal Ethics Committee in China Pharmaceutical University. All dogs were kept in separate cages and given natural light with ad libitum access to standard laboratory diet and clean water. Prior to administration, the dogs were fasted overnight and fed at 8 h post-dose. They were randomly divided into 2 experimental groups. 2-period crossover design was carried out for the experiment with a wash-out period of 1 week long. After an oral administration of 1 test capsule or one reference capsule (containing 500 mg acetazolamide) to each dog with 30 mL water, approximately 2.5 mL of blood samples were collected from the forelimb vein into heparinized tubes at the following time points: 0 h (before dosing) and 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 30, 36 h after dosing. Plasma samples were then separated by centrifugation at 4 000 × g for 5 min and kept at –75 °C until analysis.

Data analysis

The pharmacokinetic parameters were processed by non-compartmental analysis using DAS (Drug and statistics) 2.1.1 software (Mathematical Pharmacology Professional Committee of China).

Results

Method validation

A full method validation was performed according to the US FDA guidelines [21].

Specificity and selectivity

The specificity was assessed by analyzing 6 different batches of blank beagle plasma. Fig. 3 shows the typical MRM chromatograms of blank beagle plasma (Fig. 3a), spiked plasma with acetazolamide at the lower limit of quantification (LLOQ) and IS (Fig. 3b), and plasma sample obtained from beagle dogs at 1 h after oral administration (Fig. 3c). The results suggested that there was no significant interference on the determination of acetazolamide and IS from beagle plasma, and this method showed good specificity and selectivity for the following pharmacokinetic study. Under the conditions of this experiment, the retention times of acetazolamide and IS were 2.86 min and 2.92 min, respectively.

Linearity and sensitivity

The calibration curves were prepared at the concentration levels of 0.2, 0.5, 1, 2, 5, 10, 20, 50 μg/mL for acetazolamide in beagle plasma and were constructed by plotting peak area ratios of
acacetazolamide to IS against concentrations of acetazolamide with a weight of 1/\(x^2\) (\(x\) represents the concentration of acetazolamide). The typical calibration curve equation obtained was 

\[ Y = 0.000675x - 0.00593 \]  

(\(Y\) represented the peak area ratio of acetazolamide and IS), the correlation coefficient \(r^2\) was 0.9990 with variations of each concentration within ±6%, and the corresponding standard curve for acetazolamide determination was shown in Fig. 4. This method was sensitive enough to investigate the pharmacokinetics of AEC when the LLOQ was defined to be 0.2 μg/mL, and the precision (RSD=1.8%) and accuracy (93.8%–98.4%) values at the LLOQ were all within the acceptable range.

**Precision and accuracy**

5 replicates of the 3 QC concentration levels (0.5, 5, 40 μg/mL) were analyzed to evaluate the intra-day precision and accuracy, the QC samples were prepared according to the method in “Sample preparation”. The same procedure was performed once a day in 3 consecutive days to determine the inter-day precision. Accuracy was the ratio of measured concentration to theoretical concentration. The relative standard deviation (RSD) and the relative error (RE) of the concentration data obtained should be within ±20% for low QC concentrations and within ±15% for middle and high QC concentrations.

The results of precision (intra- and inter-day precision) and accuracy were summarized in Table 1. The RSD values of intra- and inter-day precision were all less than 5%, and accuracy results can meet the demands of determination.

**Matrix effect and absolute recovery**

To investigate the matrix effect, peak area of post-preparative blank plasma sample spiked with acetazolamide at 3 QC concentration levels were compared to that of standard solutions which were directly diluted by mobile phase. The results showed that the developed LC-MS/MS method can be successfully applied to the pharmacokinetic study of acetazolamide in beagle plasma.

**Stability**

The stability of acetazolamide in plasma under various conditions was evaluated. Three-level QC samples were stored at room temperature for 4 h before preparation and kept at 4 °C for 24 h after preparation. The QC samples also underwent three freeze (−75 °C)–thaw cycles and long-term freezing (−75 °C) condition for 30 days. The results of Table 2 showed that plasma samples were stable under all storage conditions described.

**Pharmacokinetic study**

In pharmacokinetic study, 6 healthy beagles were randomly divided into 2 groups, and a randomized, 2-way cross-over experiment was carried out to determine the relative bioavailability of AEC (containing 500 mg of acetazolamide per capsule). The drug concentration vs. time profile was determined after oral administration of acetazolamide extended-release capsules. The results showed that the developed LC-MS/MS method can be successfully applied to the pharmacokinetic study of acetazolamide in beagle dogs. The mean plasma concentration-time profiles of acetazolamide were shown in Fig. 5. DAS 2.1.1 software was used for the pharmacokinetic analysis, and main pharmacokinetic parameters including the maximum plasma concentration (\(C_{\text{max}}\)), time to \(C_{\text{max}}\) (\(t_{\text{max}}\)), elimination half-life (\(t_{1/2}\)), area under the curve from 0 h to infinity (\(AUC_{0-\infty}\)) and from 0 h to 36 h (\(AUC_{0-36h}\)) were calculated with non-compartment model. The results were listed in Table 4. Acetazolamide in 500 mg capsule (test and reference formulations) reached peak concentrations of 28.20 ± 4.93 μg/mL and 27.98 ± 4.92 μg/mL at 2.67 ± 0.82 h and 3.00 ± 0.63 h, respectively; \(t_{1/2}\) were 7.11 ± 3.60 h and 6.76 ± 3.64 h, respectively. \(AUC_{0-36h}\) were 211.99 ± 35.63 μg·h·mL\(^{-1}\) and 206.53 ± 34.91 μg·h·mL\(^{-1}\), respectively. \(AUC_{0-\infty}\) were 220.94 ± 42.65 μg·h·mL\(^{-1}\) and 214.87 ± 40.78 μg·h·mL\(^{-1}\), respectively. According to the values of \(AUC_{0-36h}\) and \(AUC_{0-\infty}\), the relative bioavailability of test formulation was 102.92 ± 6.65 % and 103.25 ± 8.97 %, respectively.

**Discussion**

Optimization of MS/MS parameters and chromatographic separation conditions

To avoid the interference from plasma and further improve selectivity, tandem mass spectrometry is a more preferable

**Table 1**  
Precision and accuracy for determination of acetazolamide in beagle plasma.

<table>
<thead>
<tr>
<th>Nominal concentration (μg/mL)</th>
<th>Measured concentration (μg/mL) ± SD</th>
<th>Precision RSD (%)</th>
<th>Accuracy RE (%)</th>
<th>Measured concentration (μg/mL) ± SD</th>
<th>Precision RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>0.47 ± 0.01</td>
<td>2.2</td>
<td>−5.3</td>
<td>0.47 ± 0.006</td>
<td>1.2</td>
</tr>
<tr>
<td>5.00</td>
<td>4.78 ± 0.15</td>
<td>3.2</td>
<td>−4.4</td>
<td>4.62 ± 0.14</td>
<td>3.1</td>
</tr>
<tr>
<td>40.0</td>
<td>40.06 ± 0.11</td>
<td>0.3</td>
<td>0.2</td>
<td>42.03 ± 2.22</td>
<td>5.3</td>
</tr>
</tbody>
</table>
method. In the present study, MS/MS detection was performed and electrospray ionization (ESI) source was adopted. In order to obtain the best MS/MS conditions for the detection of the analytes, standard solutions (1.0 μg/mL) of acetazolamide and IS were directly injected into mass spectrometer by a syringe pump and scanned under positive and negative MRM mode, respectively. And the negative mode showed higher ion intensities for both analytes. Under the full-scan mode in Q1 mass, the ion spray voltage, nebulizer gas, auxiliary gas, curtain gas and declustering potential were all tuned according to the ion signal response to achieve the highest selectivity and stability. Finally, the deprotonated molecule [M-H]−, m/z 220.9 for acetazolamide and m/z 248.9 for IS were selected as the parent ions. Then the product ion scan was performed, the parent ions were introduced to the Q2 mass spectrometer, and the MS/MS fragmentations resulted in product ions m/z 83.3 for acetazolamide and m/z 185.0 for IS with the optimum collision energy set of −28 V and −27 V, respectively. After collision-activated dissociation (CAD) using nitrogen as collision gas and further optimization of the MS/MS parameters in MRM mode, the ion transitions of m/z 220.9→83.3 and m/z 248.9→185.0 with the highest selectivity and ion response (>2.0×105) were chosen for the detection of acetazolamide and IS, respectively.

During the process of optimizing the chromatographic conditions, in order to achieve high resolution, shorter run time and better peak shapes, various compositions and proportions of mobile phase have been investigated. At the very start, isocratic elution by methanol and water was tried, while the peak shapes were not symmetrical, sharp or smooth. The retention times of both analytes were relatively long. Then, gradient elution was adopted, and the peak shapes were better improved, but the smoothness was still not so satisfying. Considering this situation, the organic phase was replaced by acetonitrile because its elutive power was higher than methanol, and then the analytes could be more sufficiently separated from water-soluble impurities and eluted within proper time, thus obtaining smooth and sharp peak shapes without interference and higher ion response as well as shortening the whole analysis period. The retention behaviors of analytes on different chromatographic columns have also been investigated. After comparison, Shimadzu VP-ODS C18 chromatographic column was found to be the best choice for the separation of analytes because of its low matrix effects and high column efficiency. At the injection volume of 10μL, the peak shapes of analytes turned out to be asymmetric, this phenomenon was likely caused by sample overload. After reducing the sample injection volume to 5μL, the peak shapes of analytes resulted to be more symmetric and beautiful.

Finally, a gradient elution on Shimadzu VP-ODS C18 chromatographic column with acetonitrile and water as the mobile phase at a flow rate of 0.35 mL/min was proved to be the most appropriate condition that could achieve the highest separation efficiency. Compared with other publications [14–20] involving LC-MS/MS method, the determination of acetazolamide under the optimized LC-MS/MS conditions in this study could achieve better resolution and higher ion response, shorter retention times and especially perfect peak shapes. The high MS/MS ion intensity and effective chromatographic separation could not only facilitate the whole analytical process, but also provide more accurate results with minimum interference of endogenous materials form plasma on the separation and detection of the analytes.

Table 2 Absolute recovery of acetazolamide in beagle plasma (n = 5).

<table>
<thead>
<tr>
<th>Nominal concentration (μg/mL)</th>
<th>Absolute recovery (mean ± SD, %)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>97.5 ± 2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>5.0</td>
<td>101.7 ± 2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>40.0</td>
<td>97.5 ± 3.1</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Table 3 Stability of acetazolamide in beagle plasma (n = 5).

<table>
<thead>
<tr>
<th>Nominal concentration (μg/mL)</th>
<th>Short-term storage conditions</th>
<th>Measured concentration (mean ± SD) (μg/mL)</th>
<th>Freeze-thaw condition</th>
<th>Long-term storage condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-preparative samples (room temperature, 4 h)</td>
<td>Post-preparative samples (4°C, 24 h)</td>
<td>Three freeze (−75°C)-thaw cycles</td>
<td>Freezing (−75°C, 30 d)</td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------------------------</td>
<td>------------------------------------------</td>
<td>-----------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>0.50</td>
<td>0.46 ± 0.01</td>
<td>0.46 ± 0.01</td>
<td>0.46 ± 0.01</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>5.00</td>
<td>4.38 ± 0.07</td>
<td>4.53 ± 0.11</td>
<td>4.48 ± 0.10</td>
<td>4.86 ± 0.04</td>
</tr>
<tr>
<td>40.0</td>
<td>42.38 ± 0.66</td>
<td>43.10 ± 0.37</td>
<td>38.18 ± 0.87</td>
<td>37.74 ± 0.96</td>
</tr>
</tbody>
</table>

Fig. 5 Mean plasma concentration-time curves over 36 h of acetazolamide following a single oral dose of 500 mg test and reference formulations to beagles, respectively (Each point represents the mean ± SD, n = 6).

Table 4 Main pharmacokinetic parameters of acetazolamide in plasma after oral administration of 500 mg test and reference formulations to beagle dogs, respectively.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Test formulation</th>
<th>Reference formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (μg/mL)</td>
<td>28.20 ± 4.93</td>
<td>27.98 ± 4.92</td>
</tr>
<tr>
<td>tmax (h)</td>
<td>2.67 ± 0.82</td>
<td>3.00 ± 0.63</td>
</tr>
<tr>
<td>t1/2 (h)</td>
<td>7.11 ± 3.60</td>
<td>6.76 ± 3.64</td>
</tr>
<tr>
<td>AUC0–24h (μg/mL/h)</td>
<td>211.99 ± 35.63</td>
<td>206.53 ± 34.91</td>
</tr>
<tr>
<td>AUC0–∞ (μg/mL/h)</td>
<td>220.94 ± 42.65</td>
<td>214.87 ± 40.78</td>
</tr>
</tbody>
</table>

Li X et al. LC-MS/MS Analysis of Acetazolamide... Drug Res 2014; 64: 499–504
Selection of IS
Sulfadiazine was selected as the internal standard in this method mainly because of its high ionization efficiency under ESI source and similar chromatographic separation properties with acetazolamide, so that the optimization process could be unified and efficient. Moreover, sulfadiazine can maintain its stability during sample preparation and the whole determination process.

Sample pretreatment
In pharmacokinetic studies, sample pretreatment method is crucial since errors caused by operation and low recovery usually can be introduced in this section. The most common sample preparation method for plasma acetazolamide was liquid-liquid extraction (LLE) in the earlier publications [9,10] of which the developed sample pretreatment methods were not only complex, but also time-consuming. While in this study, sample pretreatment could be simplified due to the improved sensitivity and selectivity of our current established LC-MS/MS conditions under which the protein precipitation method could also meet the requirement for acetazolamide determination and obtain higher absolute recovery. As a result, protein precipitation adopted in the current study was not only rapid and convenient but also had good reproducibility, thus simplifying and speeding up the entire pharmacokinetic study.

Method application
As obviously can be seen in Fig. 5, the plasma concentration-time profiles of acetazolamide in test and reference formulation were almost consistent and there was also no significant statistical difference between each primary pharmacokinetic parameter of the two formulations. Thus, we can conclude that with similar pharmacokinetic behaviors, test formulation was proved to be of the two formulations. Thus, we can conclude that with similar pharmacokinetic behaviors, test formulation was proved to be.

Conclusions
A simple, practical and accurate LC-MS/MS method for the determination of plasma acetazolamide has been established and validated. And the pharmacokinetic behavior of acetazolamide after oral administration of AEC to beagle dogs has been successfully analyzed by using the developed method. As discussed above, the sample pretreatment process has been greatly simplified owing to the optimized LC-MS/MS conditions in current study, thus saving time and money. In summary, this method showed its highly convenience and reliability for the rapid determination of acetazolamide in high-throughput pharmacokinetic studies.

Acknowledgement
This work was supported by Jiangsu provincial promotion foundation for the key lab of drug metabolism and pharmacokinetics (NO. BM2012012).

Conflict of Interest
No conflicts of interest exist.

References
7 Okazawa H, Yamuchii H, Sugiymoto K et al. Differences in vasodilatory capacity and changes in cerebral blood flow induced by acetazolamide in patients with cerebrovascular disease. J Neurol Med 2003; 44: 1371–1378