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Quantitation of acetazolamide in rat plasma, brain tissue and cerebrospinal fluid by high-performance liquid chromatography

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Abstract

A simple and sensitive high-performance liquid chromatographic method for the analysis of acetazolamide (AZ) in rat blood (plasma/serum, whole blood and serum ultrafiltrate), brain tissue and cerebrospinal fluid (CSF) was described. Quantitative extraction of AZ with ethyl acetate from both buffered plasma and brain tissue homogenate (pH 8.0) was achieved. Each extract was evaporated to dryness and the residue was chromatographed on a reversed-phase column. CSF was directly analysed without extraction step. The limits of detection were 0.05 μg ml^{-1} for plasma, 0.02 μg g^{-1} for brain tissue and 0.004 μg ml^{-1} for CSF. Calibration curves were linear over the working ranges of 0.1–100 μg ml^{-1} for plasma, 0.05–50 μg g^{-1} for brain tissue and 0.025–50 μg ml^{-1} for CSF. The reproducibility of AZ assay in the rat biologic media indicated very low relative standard deviations (RSDs). The recoveries of AZ added to plasma and brain tissue were more than 96% with an RSD of less than 5%. The present method was applied to studies of plasma concentration profiles of the drug after administration and its distribution into central nervous system.

Keywords: HPLC; Acetazolamide; Plasma; Brain tissue; Cerebrospinal fluid; Rat

1. Introduction

Acetazolamide (AZ), *N*-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)acetamide, is a carbonic anhydrase inhibitor (CAI), mainly used to reduce intraocular pressure in the treatment of glaucoma including its long-term management. However, the potential therapeutic value of AZ as well as other CAIs frequently has been limited by an unacceptably high incidence of side effects associated with its continued use [1]. In a previous paper, 44 out of the 92 patients in CAI therapy complained of a symptom complex of systemic adverse reactions, such as malaise, fatigue, weight loss, depression, anorexia and loss of libido [2]. Since AZ is predominantly excreted unchanged by the kidney [3], this drug would be subject to change in the pharmacokinetics at the disease states such as renal dysfunction. Therefore, it is important to study the pharmacokinetics of AZ in both normal and

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pathological condition. Furthermore, this drug has also been used for the treatment of epilepsy because of its anticonvulsive properties [1]. Nevertheless, there are very few pharmacokinetic data, especially in central nervous system (CNS), reported on AZ. Various methods using a high performance liquid chromatography (HPLC) have been published for the determination of AZ in biological samples [4–9]. However, none of these methods included the analysis of AZ in brain tissue and cerebrospinal fluid (CSF).

The aim of this study was to establish a simple and sensitive HPLC procedure for determination of AZ in rat plasma, brain tissue and CSF, and to apply the method to a pharmacokinetic study of the drug. Furthermore, we were able to determine AZ as both total and unbound fractions in plasma/serum for the study of AZ distribution to the CNS.

2. Experimental

2.1. Apparatus

A Shimadzu LC-10AD high-performance liquid chromatograph (Kyoto, Japan) was used, equipped with a Shimadzu SPD-10A UV spectrophotometric detector operating at 266 nm, and a reversed-phase column (150 mm × 4.6 mm I.D.) packed with Wakosil-II 5C18, particle size 5 μm (Wako Pure Chemical Industries, Osaka, Japan). The chromatographic data were calculated with a Shimadzu C-R7A plus data module.

2.2. Chemicals

AZ and sulfadiazine (internal standard) of analytical grade were purchased from Sigma (St. Louis, MO). Acetonitrile and ethyl acetate of liquid chromatographic grade were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other reagents were of analytical grade.

2.3. Animals and drug treatments

Male Wistar rats (10 weeks old, Nippon SLC, Hamamatsu, Japan), cannulated into the right jugular vein, were used. A bolus dose of 10 or 30 mg kg^{-1} of AZ was administered via the cannula to the rats. After the drug injection, blood was withdrawn periodically from the cannula into the heparinized tube. Plasma was immediately separated by centrifugation. At the last time of blood sampling, each rat was lightly anaesthetized with ether. CSF and blood were obtained by cisternal puncture and through the cannula, respectively. A portion of the blood was haemolyzed with an equal volume of distilled water for the whole blood assay. The serum was immediately separated from the blood by centrifugation with the serum separator, Fibrichin (Takazono Sangyo, Osaka, Japan) and a portion of the serum was ultrafiltered by using MPS-3 (cut-off molecular weight 30000; Amicon, Beverly, MA). In the preliminary study, it was confirmed that no adsorptive losses of AZ to the membrane of the device occurred during ultrafiltration. Immediately after CSF and final blood collection, the rat was sacrificed by microwave irradiation focused on its head for 0.9 s using a microwave applicator, Toshiba Model TMW-6402A (Tokyo, Japan). Each rat was decapitated and the whole brain was eased out of the skull, and the cerebellum and brain stem were removed. The brain was weighed after careful removal of the dural and subarachnoidal vessels. Drug-free (i.e. blank) blood plasma, CSF and brain tissue were also obtained from the control rats which were injected with solvent alone. The biological specimens were kept frozen at below −20°C protected from light until the analysis and AZ in these biological samples was completely stable for 2 months.

The animal experiments were done in accordance with the procedures outlined in the guidelines for care and use of laboratory animals from the Committee of Shimane Medical University. Animals were housed in an environmentally controled room of constant temperature and humidity and dark/light (12/12 h) cycle.

2.4. Standard solutions

The stock solution of AZ (1 mg ml^{-1}) or the internal standard (sulfadiazine, 300 μg ml^{-1}) was prepared by dissolving each drug first in a small volume (less than 0.15 ml for 10 mg of any drug) of 1 M sodium hydroxide and by diluting the solution with the distilled water. Each solution was kept frozen at below −20°C protected from light and was completely stable for 2 months. Working standard solution and internal standard solution were prepared by diluting the stock solution with distilled water.

2.5. Analytical procedures

To 50 μl of rat plasma/serum, whole blood (100 μl of hemolysate) or 100 μl of serum ultrafiltrate samples were added 0.5 ml of phosphate buffer consisting of 0.1 M disodium hydrogenphosphate adjusted to pH 8.0 with 0.1 M potassium dihydrogenphosphate, 1 g of ammonium sulfate and 100 μl of the internal standard solution (3 μg ml^{-1} of sulfadiazine). This mixture was extracted with 3.0 ml of ethyl acetate in a 10-ml glass tube, which was shaken vigorously for 15 min. After centrifugation at 1500 g for 10 min at ambient temperature, 2.0 ml of the organic phase was transferred to another tube and evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The residue was reconstituted in 200 μl of 0.05 M glycine buffer (pH 10.0) under vigorous shaking for 10 min. A 20-μl aliquot was injected into the chromatograph.

Each brain tissue was homogenized in three volumes (v/w) of ice-cold normal saline using a Teflon homogenizer. To a portion (0.5 ml) of the homogenate, 0.5 ml of 0.1 M phosphate buffer (the same as described in the plasma extraction procedure), 100 μl of the internal standard solution (3 μg ml^{-1} of sulfadiazine) and 3 ml of diethyl ether were added. The solution was shaken for 5 min and centrifuged at 1000 g for 10 min at 4°C. The ether phase was discarded. To the aqueous phase, 1 g of ammonium sulfate and 5 ml of ethyl acetate were added. The resultant mixture was shaken for 15 min and centrifuged at 1500 g for 10 min at ambient temperature. An aliquot (4 ml) of the organic phase was evaporated to dryness under a gentle stream of dry nitrogen at 40°C. The residue was reconstituted with 0.05 M glycine buffer (pH 10.0) under vigorous shaking for 15 min and filtered through a membrane filter (0.45 μm, SPIN-X®, Corning Costar, Cambridge, MA). A 20-μl aliquot of the filtrate was used for injection onto the HPLC column.

To 30 μl of CSF, 30 μl of 0.05 M glycine buffer (pH 10.0) containing 0.6 μg ml^{-1} of sulfadiazine was added and mixed. A 20-μl aliquot of the mixture was directly applied onto the column.

Each injected solution was completely stable at below 4°C protected from light within 2 days and all procedures of the analysis were performed within 48 h.

2.6. HPLC Conditions

The mobile phase was acetonitrile—0.05 M sodium acetate (1:9, v/v) adjusted to pH* 4.1 with glacial acetic acid. The flow-rate was 1.0 ml min^{-1} and separation was performed at 40°C.

2.7. Calibration graphs

AZ was dissolved in drug-free plasma to give a concentration range of 0.1–100 μg ml^{-1} (seven points, $n = 5$). The homogenate of drug-free brain tissue was spiked with increasing amounts of AZ in the concentration range of 0.05–50 μg g^{-1} wet tissue (eight points, $n = 5$). The calibration curve for CSF was prepared by using normal saline spiked with the drug standard solution to make the concentration range of 0.025–50 μg ml^{-1} (eight points, $n = 5$). These samples were then assayed according to the analytical procedure described above. Calibration graphs were constructed by plotting the peak-area ratio of the drug to the internal standard against the concentration.

2.8. Reproducibility

Biological samples were taken from the rats at appropriate times after the administration of AZ. Ten (plasma and brain tissue) or five (CSF) repeated analyses of biological samples were carried out.

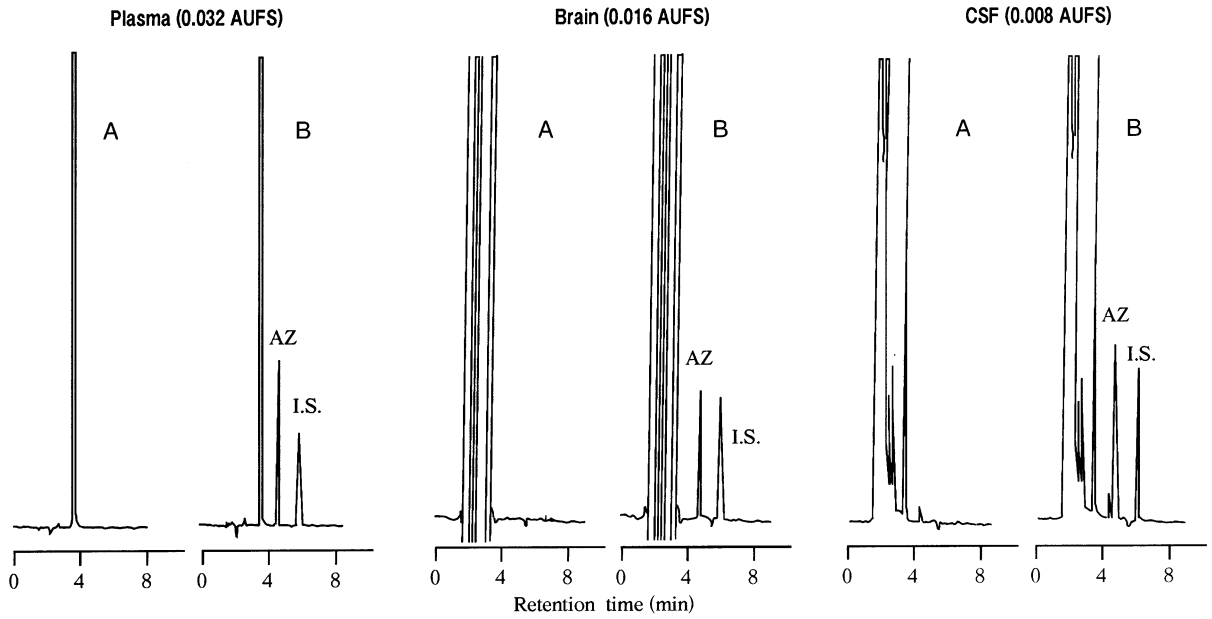

Fig. 1. Typical chromatograms of AZ obtained from rat plasma, brain and CSF. A, blank samples; B, samples obtained from rats 30 min (plasma), 5 min (brain and CSF) after intravenous administration (10 mg kg^{-1}, plasma; 30 mg kg^{-1}, brain and CSF) of AZ, I.S., internal standard (sulfadiazine).

2.9. Accuracy

The accuracy of the present method was evaluated by measuring the recovery of known amounts of AZ added to rat plasma or brain tissue homogenate. The drug-free plasma samples and brain tissue homogenates were spiked with 1 or 50 μg ml^{-1}, 0.1 or 5 μg g^{-1} of AZ, respectively. Recovery was determined by comparing the amount of the drug measured by calculating from an adequate standard with known added amount.

3. Results

Typical chromatograms resulting from HPLC analysis of AZ in plasma, brain tissue and CSF obtained from the rat are shown in Fig. 1. The chromatograms are representative for the analysis of blank samples from the drug-free rats (Fig. 1A) or plasma, brain tissue and CSF samples taken from rats receiving the intravenous administration of AZ (Fig. 1B). AZ and sulfadiazine (internal standard) were eluted at retention times of about 4.7 and 6.0 min, respectively, as well resolved peaks. No interfering endogenous peaks could be detected in any chromatogram of drug-free plasma, brain tissue or CSF.

The limits of detection of the assay determined in extracted plasma and brain tissue, and in CSF (without extraction), which were defined as a signal-to-noise ratio of 3:1, were 0.05, 0.02 and 0.004 μg ml^{-1}, respectively.

Calibration curves were generated by a least-squares linear regression analysis using the peak area ratio against drug concentrations. Satisfactory linearity was observed in the ranges of 0.1–100 μg ml^{-1} for plasma, 0.05–50 μg g^{-1} for brain tissue and 0.025–50 μg ml^{-1} for CSF. The regression equations by the method of least-squares were $y = 0.234\ (\pm 0.002)\ x + 0.020\ (\pm 0.008)$, $r^2 = 0.9998$ for plasma; $y = 0.258\ (\pm 0.003)\ x + 0.020\ (\pm 0.010)$, $r^2 = 0.9995$ for brain tissue; and $y = 1.019\ (\pm 0.002)\ x - 0.006\ (\pm 0.003)$, $r^2 = 0.9999$ for CSF, where y is the peak area ratio of the drug to the internal standard, x is the concentration in plasma (μg ml^{-1}),

Table 1
Precision of the assay of AZ in rat plasma, brain tissue and CSF collected following bolus intravenous administration

Sample	AZ dose (mg/kg)	Time after dosing (min)	AZ level[b,c]	RSD (%)
Plasma	10	60	6.89 ± 0.08	3.5
	10	5	35.2 ± 0.4	3.5
Brain	10	90	0.057 ± 0.001	4.0
	30	5	2.58 ± 0.03	3.5
CSF	10	—[a]	0.177 ± 0.002	3.1

RSD, relative standard deviation.
[a] Pooled sample collected at appropriate times after administration (3–60 min).
[b] Mean ± S.E. of ten (plasma and brain) or five (CSF) determinations.
[c] Plasma, CSF (μg ml^{-1}), Brain (μg g^{-1}).

brain tissue (μg g^{-1}) or CSF (μg ml^{-1}) and r^2 is the determination coefficient (adjusted by using the degrees of freedom). Each value for the regression slope or intercept was expressed as the estimated value with ± standard error.

Table 1 shows the precision of this assay method of AZ in plasma, brain tissue and CSF. Each assay had a relative standard deviation (RSD) below 4% at the concentration tested.

Table 2 shows the recovery data for AZ spiked to drug-free plasma and brain tissue homogenate at two different concentrations. The recoveries for AZ were 97.2 and 96.1% at the concentrations of 1 and 50 μg ml^{-1} in plasma, 105.7 and 96.8% at the concentrations of 0.1 and 5 μg g^{-1} in brain tissue, respectively, with the RSD less than 5% in all cases.

The present method was then used in the preliminary pharmacokinetic study on AZ. Fig. 2 shows the plasma concentration versus time data of AZ after bolus intravenous administration of 10 mg kg^{-1} of AZ to rats. The plasma total concentrations of AZ showed a bi-exponential decline with time. The elimination half-life at the terminal phase was about 20 min. The bound fraction of AZ to serum protein, which was calculated from the total and unbound concentrations of AZ in the serum sample, was about 80% at 60 min after the administration. Fig. 3 shows AZ concentrations in brain tissue and CSF and ratios to the unbound concentration of AZ in serum at 60 min following bolus intravenous administration of 10 mg kg^{-1} of AZ to rats. The mean AZ

Table 2
Accuracy of the assay of AZ in rat plasma and brain tissue

Sample	Added[a]	Found[a,b]	Recovery[a] (%)	RSD (%)
Plasma	1.0	0.972 ± 0.010	97.2 ± 1.0	2.3
	50.0	48.0 ± 0.2	96.1 ± 0.4	1.8
Brain	0.1	0.106 ± 0.002	105.7 ± 2.2	4.5
	5.0	4.84 ± 0.08	96.8 ± 1.6	3.8

[a] Plasma (μg ml^{-1}), Brain (μg g^{-1}).
[b] Mean ± S.E. of five determinations.

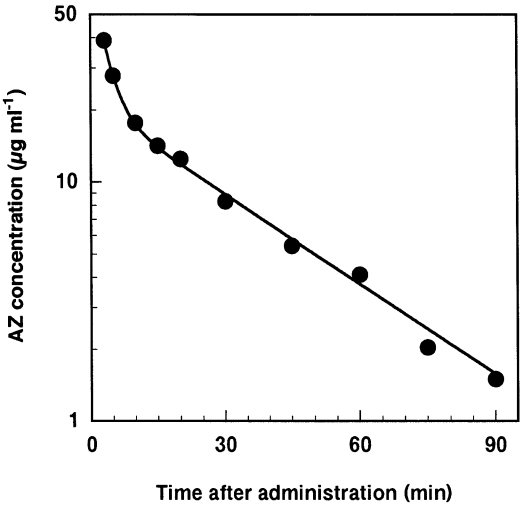

Fig. 2. Concentration-time profile of AZ in plasma following an intravenous bolus administration of 10 mg kg^{-1} to rats. Each point represents the mean of two rats. The line represents the computer-fitted biexponential curve for the mean data (Weight(i) = Ci^{-1}, where C was the plasma drug concentration) using a computer program, WinNonlin®.

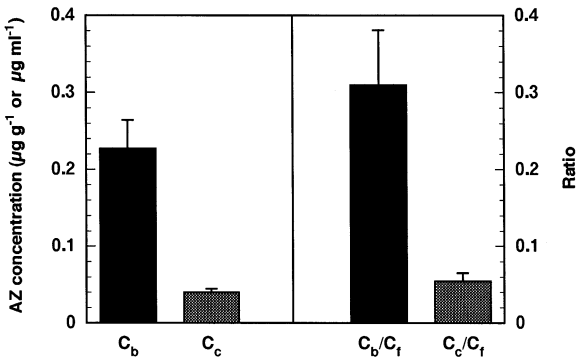

Fig. 3. Brain tissue and CSF concentrations of AZ and ratios to the unbound concentration of AZ in serum at 60 min following an intravenous bolus administration of 10 mg kg^{-1} to rats. C_b, brain tissue concentration (μg g^{-1}); C_c, CSF concentration (μg ml^{-1}); C_f, serum unbound concentration (μg ml^{-1}). Each column and vertical bar represent the mean and standard deviation of six rats. C_b was the net AZ concentration in the brain parenchyma which was corrected by the measured whole blood drug concentration and the calculated cerebral blood volume.

concentration in brain tissue and the ratio to the unbound concentration in serum were much higher than those of the CSF.

4. Discussion

In earlier studies, several methods have been described for the assay of AZ in biologic media, i.e. in plasma, whole blood, saliva or urine [10–13]. These included a colorimetric method and an enzymatic method. More recently, several gas-chromatographic methods have been reported [14,15]. However, HPLC technique has replaced these procedures because of its higher sensitivity and precision. Various HPLC methods have been developed for the determination of AZ in biological samples [4–8]. However, these HPLC procedures require relatively large amount of biological samples [4–6] and complicated steps such as repeated solvent extraction procedures [4,5]. Furthermore, the internal standard compounds employed in some of the previous papers were not commercially available [4,7,8]. Chapron and White reported a HPLC assay method including a back extraction of AZ in human blood and urine [9]. Although their HPLC method has not been applied to other biological samples, it was considered to be utilized in laboratory animals such as rats because of its small initial volumes (200 μl) of the samples. Therefore, we first attempted to apply the method of Chapron and White to AZ determination in rat blood, brain tissue and CSF. As the results, their method showed too low absolute recoveries for these samples to detect AZ sensitively. Additionally, relatively large interfering peaks were found on the chromatograms for brain tissue samples, probably owing to accumulation of several endogenous substances during the back extraction procedure. Consequently, we have modified the clean-up procedure [9] in order to determine AZ in a small volume of the biological specimen.

The proposed method could be adapted for smaller size of plasma, brain tissue and CSF samples such as less than 50 μl, 125 mg and 30 μl, respectively. This method involves the solvent extraction and concentration procedures for the plasma and brain tissue samples and the direct injection of CSF samples without extraction after dilution with an equal volume of the internal standard solution. AZ was known to be entirely excreted unchanged by the kidney [3]. Appreciable interferences of neither endogenous constituents nor metabolites were detected with the peak area determination of AZ as well as the internal standard. Since AZ concentrations as low as 0.1 μg ml^{-1} in plasma, 0.05 μg g^{-1} in brain tissue or 0.025 μg ml^{-1} in CSF can be measured using this method, it was expected to be applicable to the detailed study of AZ disposition kinetics in plasma, brain tissue and CSF. The satisfactory reproducibility of this assay method for AZ in rat biologic media was indicated by very low RSDs in the repeated analysis of the samples. In the recovery test, the measured amounts of AZ in the spiked plasma or the brain tissue homogenate yielded more than 96% of the known amount with a RSD of less than 5%. There was no problem with the recovery of AZ in CSF itself. Both reproducibility and recovery in the determination of AZ were satisfactory over a wide range of concentration.

Therapeutic plasma concentration for AZ has been reported to range from 5 to 15 μg ml^{-1} in human [16,17]. Our animal study at 10 mg kg^{-1} dose, that was under the maximum recommended dose for human adults (1000 mg a day), showed a reasonable plasma concentration-time profile (Fig. 2). Since the determination limit of this assay method in plasma, 0.1 μg ml^{-1}, was about 500-fold lower than the initial concentration of AZ in plasma, the present method was found to be sufficiently sensitive for the pharmacokinetic analysis of AZ in plasma. Although the brain tissue and CSF levels were much lower than that of the plasma at 60 min following the intravenous dosing, both AZ concentrations were able to be determined by this HPLC method (Fig. 3). Results of the detailed CNS pharmacokinetics for AZ from both normal and pathological rats will be reported later.

5. Conclusions

The present HPLC method for the determination of AZ in rat plasma, brain tissue and CSF provides a sufficiently sensitive, accurate and reproducible analytical procedure. It is expected that this method will be used successfully in kinetic studies of AZ in the blood and CNS.

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