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GC-NICI-MS analysis of acetazolamide and other sulfonamide (R-SO$_2$-NH$_2$) drugs as pentafluorobenzyl derivatives [R-SO$_2$-N(PFB)$_2$] and quantification of pharmacological acetazolamide in human urine

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Abstract

Acetazolamide (molecular mass (MM), 222) belongs to the class of sulfonamides (R-SO$_2$-NH$_2$) and is one of the strongest pharmacological inhibitors of carbonic anhydrase activity. Acetazolamide is excreted unchanged in the urine. Here, we report on the development, validation and biomedical application of a stable-isotope dilution GC-MS method for the reliable quantitative determination of acetazolamide in human urine. The method is based on evaporation to dryness of 50 µL urine aliquots, base-catalyzed derivatization of acetazolamide (d$_0$-AZM) and its internal standard [acetylo-$^2$H$_3$]acetazolamide (d$_3$-AZM) in 30 vol% pentafluorobenzyl (PFB) bromide in acetonitrile (60 min, 30 °C), reconstitution in toluene (200 µL) and injection of 1-µL aliquots. The negative-ion chemical ionization (NICI) mass spectra (methane) of the PFB derivatives contained several intense ions including [M]$^-$ at m/z 581 for d$_0$-AZM and m/z 584 for d$_3$-AZM, suggesting derivatization of their sulfonamide groups to form N,N-dipentafluorobenzyl derivatives (R-SO$_2$-N(PFB)$_2$), i.e., d$_0$-AZM-(PFB)$_2$ and d$_3$-AZM-(PFB)$_2$, respectively. Quantification was performed by selected-ion monitoring of m/z 581 and 83 for d$_0$-AZM-(PFB)$_2$ and m/z 584 and 86 for d$_3$-AZM-(PFB)$_2$. The limits of detection and quantitation of the method were determined to be 300 fmol (67 pg) and 1 µM of acetazolamide, respectively. Intra- and inter-assay precision and accuracy for acetazolamide in human urine samples in pharmacologically relevant concentration ranges were determined to be 0.3 – 4.2% and 95.3 - 109%, respectively. The method was applied to measure urinary acetazolamide excretion after ingestion of a 250 mg acetazolamide-containing tablet (Acemit®) by a healthy volunteer. Among other tested sulfonamide drugs, methazolamide (MM, 236) was also found to form a N,N-dipentafluorobenzyl derivative, whereas dorzolamide (MM, 324) was hardly detectable. No GC-MS peaks were obtained from the PFB bromide derivatization of hydrochlorothiazide (MM, 298), xipamide (MM, 355), indapamide and metholazone (MM, 366 each) or brinzolamide (MM, 384). We demonstrate for the first time that sulfonamide drugs can be derivatized with PFB bromide and quantitated by GC-MS. Sulfonamides with
MM larger than 236 are likely to be derivatized by PFB bromide but to lack thermal stability.

**Keywords:** Acetazolamide; Derivatization; Quantification; Sulfonamides; Validation

1. **Introduction**

   Acetazolamide (\(\text{N}-(5\text{-sulfamoyl-1,3,4-thiadiazol-2-yl})\text{acetamide}\), MM 222.25; CAS number 216665-38-2; see Figure S1, Supplement) is one of the oldest therapeutically used diuretic drugs. Acetazolamide is a strong inhibitor of carbonic anhydrase (CA) which catalyzes the reversible reaction between \(\text{CO}_2\) and \(\text{H}_2\text{O}\) to form bicarbonate and protons (\(\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+\)) [1]. Acetazolamide and other sulfonamide (R-SO_2-NH_2) drugs (see Figure S1) are widely used for the treatment of glaucoma, mountain sickness, sleep apnea, epilepsy and hypertension [1]. The pharmacological action of acetazolamide and relatives in several organs such as eyes, brain and kidney is based on the inhibition of CA activity. In glaucoma and epilepsy, inhibition of CA activity by acetazolamide results in reduction of intraocular and cerebral pressure. Inhibition of CA activity in the kidney results in enhanced excretion of \(\text{HCO}_3^-\) and inorganic nitrite (NO\(_2^-\)) [2,3]. Acetazolamide-induced loss of nitrite may be of particular importance because NO\(_2^-\) is considered an abundant reservoir of the endogenous potent vasodilator nitric oxide (NO). The urinary nitrate-to-nitrite molar ratio, i.e., \(U_{\text{NO}_xR}\), may be a useful measure of renal CA-dependent reabsorption of urinary nitrite in humans [4]. Besides its therapeutic use, acetazolamide gained importance as a potential performance-enhancing drug in sport doping [5].

Several analytical methods based on liquid chromatography (LC) [6–12] and gas chromatography (GC) [13–21] have been reported for the determination of acetazolamide and related sulfonamides in various biological samples including plasma and urine. For GC analysis, sulfonamides such as acetazolamide require conversion of their sulfonamide group to thermally stable and volatile derivatives. The amine group of the sulfonamide group of acetazolamide and many other sulfonamides is the only functional group that is accessible for chemical derivatization. Methylation of the amine
group of sulfonamides has been often performed using methyl iodide (iodomethane, CH$_3$I) [13-19]. Silylation of the amine group of sulfonamides for GC has also been reported [20, 21].

Pentafluorobenzyl (PFB) bromide (PFB-Br) is a versatile derivatization reagent in chromatography (GC, LC) and mass spectrometry (MS) [22]. It can be used for the derivatization of nucleophilic substances and inorganic and organic anions both in aqueous phase such as plasma and urine and in anhydrous organic solvents such as acetonitrile [2, 23–25]. Analogous to the derivatization of amine groups such as those in creatinine with PFB-Br [24], we assumed that PFB-Br may be useful as a derivatization reagent for the amine group of acetazolamide and other sulfonamides in GC-based analytical methods. The reaction of R-SO$_2$-NH$_2$ with PFB-Br may lead to the formation of mono-PFB derivatives (R-SO$_2$N(PFB)) and di-PFB derivatives (R-SO$_2$-N(PFB)$_2$). To the best of our knowledge, the derivatization of acetazolamide or other sulfonamides with PFB-Br has not been reported thus far.

In the present work, we demonstrate that PFB-Br is useful for the N-alkylarylation of the sulfonamide group of acetazolamide representing the group of sulfonamides. We found that the derivatization of acetazolamide is best performed in anhydrous acetonitrile using N,N-diisopropylethylamine (i.e., Hünig base) as the catalyst under very mild conditions (30 °C) (Figure S1). As acetazolamide is excreted in the urine without metabolization, we developed and validated a GC-MS method for the quantitative determination of acetazolamide in human urine using commercially available [acetylo-$^2$H$_3$]acetazolamide as the internal standard (IS). Negative-ion chemical ionization (NICI) of the PFB derivative of acetazolamide provided the lowest limit of detection (LOD) thus far reported for acetazolamide. We also tested the utility of PFB-Br for the derivatization of other sulfonamide drugs. The applicability of the method in clinical-pharmacologic settings was demonstrated upon ingestion of a 250 mg acetazolamide tablet by a healthy volunteer.
2. **Experimental**

2.1. **Materials and chemicals**

[acetylo-$^2$H$_3$]Acetazolamide (d$_3$-AZM; declared amount, 1 mg) was obtained from Hycultec (Beutelsbach, Germany) and was diluted in dimethyl sulfoxide (DMSO) in the original flask. The chemical and isotopic purity of d$_3$-AZM had not been declared by the supplier. 2,3,4,5,6-Pentafluorobenzyl bromide, $N,N$-diisopropylethylamine, pentafluoropropionic anhydride (PFPA, >99%), 2,3,4,5,6-pentafluorobenzoyl chloride (>99%), unlabeled acetazolamide (d$_0$-AZM; >99%) and all the other sulfonamides (>98%) used in the present study were obtained from Sigma-Aldrich (Darmstadt, Germany). DMSO and acetonitrile (GC grade; dried over molsieve) were purchased from Merck (Darmstadt, Germany). Toluene (p.a.) was purchased from Baker (Deventer, The Netherlands). The silylation reagent $N,O$-bis(trimethylsilyl)trifluoroacetamide (BSTFA, >99%) was obtained from Macherey-Nagel (Düren, Germany).

2.2. **Safety considerations**

PFB-Br and PFPA are corrosive. PFB-Br is an eye irritant. Inhalation and contact with skin and eyes should be avoided. All work should be performed in a well-ventilated fume hood.

2.3. **Preparation of calibration standards**

Acetazolamide is freely soluble in DMSO. Solutions and dilutions of acetazolamide and all other sulfonamides were prepared in DMSO. Stock solutions of d$_0$-AZM (100 mM) and d$_3$-AZM (50 mM) and their working dilutions (each 10 mM) were stored at 8 °C and −18 °C, respectively.

2.4. **Biological samples and preparation of urine samples**

Acetazolamide-free urine samples used in method development and validation were obtained from healthy volunteers after conformed consent. These volunteers did not ingest acetazolamide at least two weeks before. Samples (1 mL aliquots) were kept
frozen at –18 °C until analysis. Prior to sample derivatization, urine samples were thawed and centrifuged (3350×g, 5 min).

In quantitative analyses, 50 µL aliquots of the clear urine supernatants were placed in 1.8 mL glass vials and 5 µL aliquots of a 10 mM solution of d₃-AZM in DMSO were added. Urine and DMSO were evaporated to dryness under a stream of nitrogen gas. To remove effectively remaining water, ethanol (100 µL) was added, the vials were vortex-mixed for about 10 s and then ethanol was evaporated to dryness under a stream of nitrogen gas.

2.5. Derivatization procedure with PFB-Br in water-free phase

Solid residues were dissolved in anhydrous acetonitrile (100 µL). Then, 10 µL of N,N-diisopropylethylamine and 10 µL of 30 vol% PFB-Br in anhydrous acetonitrile were added, the glass vials were tightly sealed and vortex-mixed for about 10 s. Subsequently, the samples were heated for 60 min at 30 °C on a thermostatic block model MBT250-4 from Kleinfeld Labortechnik (Gehrden, Germany). After cooling to room temperature the samples were evaporated again to dryness under a stream of nitrogen gas and the residues were reconstituted with 200 µL aliquots of toluene and vortexed for 2 min. Clear and particle-free aliquots (about 180 µL) of the toluene phases were transferred into 1.8 mL autosampler glass vials equipped with 200 µL microinserts for GC-MS analysis as described below.

2.6. Validation of the method

The GC-MS method was validated for acetazolamide in urine samples donated by three healthy humans. Pharmacologically relevant concentrations ranges (0 – 1000 µM acetazolamide) were used. Validation experiments included linearity, accuracy (recovery, %) and precision (RSD, %). For the sake of clarity, the validation experiments are described in detail in the sections Results and Discussion.
2.7. GC-MS conditions

GC-MS analyses were performed on a single-quadrupole mass spectrometer model ISQ directly interfaced with a Trace 1310 series gas chromatograph equipped with an autosampler AS 1310 from ThermoFisher (Dreieich, Germany). The gas chromatograph was equipped with a 15 m long fused-silica capillary column Optima 17 (0.25 mm I.D., 0.25 µm film thickness) from Macherey-Nagel (Düren, Germany). In quantitative analyses, the following oven temperature program was used with helium (at a constant flow rate of 1 mL/min) as the carrier gas: 1.0 min at 90 °C, then increased to 250 °C at a rate of 35 °C/min and to 320 °C at a rate of 35 °C/min, respectively. The column was held at 320 °C for 6 min. Interface, injector and ion-source were kept constant at 260 °C, 200 °C and 250 °C, respectively. Electron energy was set to 70 eV and electron current to 50 µA. Methane (2.4 mL/min) was used as the reagent gas for NICI. Aliquots (1 µL from the toluene phase) were injected in the splitless mode by means of the autosampler. Quantification of acetazolamide was performed in the NICI mode by selected-ion monitoring (SIM) the ions with \( m/z \) 581 and \( m/z \) 83 for \( d_0 \)-AZM and \( m/z \) 584 and \( m/z \) 86 for \( d_3 \)-AZM (IS) with a dwell-time of 50 ms for each ion. The electron multiplier voltage was set to 2025 V. Deviations from the conditions described above are mentioned in the sections Results and Discussion.

2.8. Data analysis and presentation

If not otherwise specified, quantitative analyses were performed in triplicate. Unpaired \( t \) test was used to check statistical significance. Values are presented as mean±SD.

3. Results

3.1. Testing derivatization methods for acetazolamide

Acetazolamide (Figure S1) and methazolamide have a single functional group that is accessible to chemical derivatization. This is the amine group of the sulfonamide functionality; other sulfonamides such as dorzolamide possess additional derivatizable...
functional groups. We tested the utility of several derivatization agents for acetazolamide representing the group of sulfonamide drugs for GC-NICI-MS analysis. These derivatization reagents included pentafluorobenzyl bromide (PFB-Br), pentafluorobenzoyl chloride (PFBzoyl-Cl) and pentafluoropropionic anhydride (PFPA). Previously, we found these reagents to be useful for amine groups-containing substances such as creatinine [24], dimethyamine (DMA) [26] and amino acids [27], respectively.

We also tested $N,O$-bis(trimethylsilyl)trifluoroacetamide (BSTFA), which is a versatile reagent for different functionalities including hydroxyl and amine groups. In our tests, BSTFA was found to be not suitable, because derivatization (60 min, 60 °C) resulted in loss of the acetyl group in d$_{0}$-AZM and d$_{3}$-AZM, thus not allowing quantitative analysis of acetazolamide (data not shown). Procedures previously reported for the derivatization of methyl esters of amino acids [27] revealed that PFPA is not useful for the derivatization of acetazolamide (data not shown). By using a previously reported procedure for the extractive derivatization of urinary dimethylamine (DMA) with PFBzoyl-Cl [26], acetazolamide could also not be derivatized (data not shown).

PFB-Br is a versatile derivatization reagent in nucleophilic substitution reactions [22]. Its utility in GC-MS has been demonstrated for different classes of substances in aqueous and anhydrous systems [22]. We found that acetazolamide cannot be derivatized in aqueous buffered solutions including phosphate and Tris buffer of neutral pH value and in human urine (pH range, 5.5 – 7.8) (data not shown). In contrast, acetazolamide was found to be readily derivatized with PFB-Br in anhydrous acetonitrile using $N,N$-diisopropylethylamine as the base catalyst under very mild conditions and short derivatization times as has been reported for carboxylic acids including prostaglandins [28], i.e., warming the derivatization mixture for 60 min at 30 °C. Higher derivatization temperatures tested (i.e., 40 °C, 50 °C, 60 °C) were found to provide yellow-to-brownish colored derivatization solutions, most likely due to decomposition reactions (data not shown). For these reasons we selected PFB-Br derivatization of acetazolamide in anhydrous acetonitrile and tested the utility of this derivatization for methazolamide and other sulfonamide drugs.
3.2. GC-NICI-MS characterization of the PFB derivatives of sulfonamides

Each 50 nmol of d₄₀-AZM and d₃₀-AZM were mixed, derivatized with PFB-Br (60 min, 30 °C), the sample was evaporated to dryness and the residue was reconstituted with toluene as described in Experimental. The appearance of the originally clear and colorless solution did not change upon derivatization. GC-NICI-MS mass spectra were obtained by injecting 1 µL aliquots of the toluene phases (180 µL) each corresponding to 250 pmol of d₄₀-AZM and d₃₀-AZM (assuming quantitative derivatization). GC-NICI-MS analysis of the sample in scan mode resulted in few GC peaks. The mass spectrum obtained from the GC peak of the PFB derivatives of the mixture of d₄₀-AZM and d₃₀-AZM eluting at about 8.35 min is shown in Fig. 1. It contains five pairs of ions differing by 3 Da each, most likely due to the presence of the intact acetyl groups in the PFB derivatives of d₄₀-AZM and d₃₀-AZM.

The largest anions at m/z 581 and m/z 584 in the mass spectrum can be assigned to the N,N-dipentafluorobenzyl derivatives of d₄₀-AZM (R=SO₂-N(PFB)₂; molecular mass, 582) and d₃₀-AZM (R(d₃)=SO₂-N(PFB)₂; molecular mass, 585), respectively. The ions m/z 375 and m/z 357 are common to the d₄₀-AZM and d₃₀-AZM derivatives and are likely produced from breaking of the derivatized sulfonamide groups. Presumably, m/z 357 derives from m/z 375 by loss of a water molecule (H₂O, 18 Da). The ion m/z 167 is also common to d₄₀-AZM and d₃₀-AZM and is due to [C₆F₅]⁻. The ions m/z 83 and m/z 86 carry each one acetyl group and their structures are likely to be [CH₃CO-N-CN]⁻ and [CD₃CO-N-CN]⁻, respectively. The most intense and common ion in the mass spectra of d₄₀-AZM-(PFB)₂ and d₃₀-AZM-(PFB)₂ is m/z 58 and is likely to be the negatively charged thio-ethene epoxide [(CH=CH)S]⁻.

It is worth mentioning that the retention time of the PFB ester-methoxime (MO)-trimethylsilyl (TMS) ether derivative of prostaglandin E₂ (i.e., PGE₂-PFB-MO-(TMS)₂; molecular mass, 705) under the same GC-MS conditions was 8.32 min. This observation indicate that the C₃₄⁺-species PGE₂-PFB-MO-(TMS)₂ is as volatile as the C₃₈⁺-species d₄₀-AZM-(PFB)₂ and d₃₀-AZM-(PFB)₂, presumably because of its two TMS ether functionalities.
The mass spectrum obtained from the GC peak of the PFB derivative of methazolamide (retention time, 7.8 min) is shown in Fig. 2. The ions at m/z 357 and m/z 375 in this mass spectrum indicate that dorzolamide reacts with PFB-Br to form the \( N,N\)-dipentafluorobenzyl derivative \( \text{R-SO}_2-N(\text{PFB})_2 \) (molecular mass, 596) analogous to acetazolamide. However, the \( \text{R-SO}_2-N(\text{PFB})_2 \) derivative of methazolamide ionizes quite differently than the \( \text{R-SO}_2-N(\text{PFB})_2 \) derivative of acetazolamide. The intense ion at m/z 415 is likely to be due to \([\text{M-}\text{PFB}]^-\). The most intense ion m/z 220 in the mass spectrum of the \( N,N\)-dipentafluorobenzyl derivative of methazolamide is likely to be due to \([\text{M-}\text{NH(\text{PFB})}_2]^-\) which is absent in the mass spectrum of \( N,N\)-dipentafluorobenzyl derivative of acetazolamide. The reason for this disparity could be the formation of the electrically neutral \([\text{M-}\text{NH(\text{PFB})}_2]\) or radical species \([\text{M-}\text{NH(\text{PFB})}_2^*]\). A likely explanation of the remarkable differences in the NICI mass spectra of the \( N,N\)-dipentafluorobenzyl derivatives of the structurally closely acetazolamide and methazolamide and the shorter retention time of the methazolamide derivative could be the methyl group on the amidic N atom of methazolamide. This methyl group makes the derivative more lipophilic, volatile, presumably more stable, and influences the NICI as well (see Figure S2).

The mass spectrum obtained from the GC peak of the PFB derivative of dorzolamide (retention time, 10.2 min) is shown in Figure S3. The ions at m/z 357 and m/z 375 in this mass spectrum indicate that dorzolamide reacts with PFB-Br to form the \( N,N\)-dipentafluorobenzyl derivative \( \text{R-SO}_2-N(\text{PFB})_2 \) (molecular mass, 684) analogous to acetazolamide and methazolamide. Yet, in contrast to the mass spectra of the PFB derivatives of acetazolamide and methazolamide, the NICI mass spectrum of the dorzolamide derivative does not contain the anion at m/z 58 \([((\text{CH=CH})\text{S}])^-\). This could be due to the lack of the thiadiazo structure in dorzolamide.

3.3. **GC-NICI-MS characterization and standardization of \([\text{acetylo-}^2\text{H}_3]\)acetazolamide (\(d_3\)-AZM)**

Declared amounts of commercially available stable-isotope labeled analogs, especially of those delivered in very small, not (accurately) weighable amounts, may
differ from those obtained experimentally. The chemical and isotopic purity of the d$_3$-AZM used in our study had not been declared by the supplier and was, therefore, determined experimentally as follows.

Aliquots (5 µL) of separate 10 mM solutions of d$_3$-AZM and d$_0$-AZM in DMSO were derivatized and analyzed separately. Aliquots (1 µL) of the toluene phases assuming to contain 250 pmol of d$_3$-AZM or d$_0$-AZM were injected on three different days each in triplicate per day and SIM of m/z 581, m/z 584, m/z 83 and m/z 86 was performed. The peak area ratio (PAR) values for d$_3$-AZM in the absence of synthetic d$_0$-AZM were determined to be (mean±SD, n=9) 0.00257±0.001 for the PAR m/z 581 to m/z 584 and 0.00477±0.002 for the PAR m/z 83 to m/z 86. These data indicate an isotopic purity of the commercially available d$_3$-AZM preparation of about 99.7% at $^2$H and no appreciable contribution of d$_0$-AZM to d$_3$-AZM in the commercially available d$_3$-AZM. The PAR values for d$_0$-AZM in the absence of d$_3$-AZM were determined to be (mean±SD, n=9) 0.0233±0.001 for the PAR m/z 584 to m/z 581 and 0.00122±0.0007 for the PAR m/z 86 to m/z 83, indicating no appreciable contribution of d$_3$-AZM to d$_0$-AZM.

The standardization procedure of d$_3$-AZM and the linearity of the method was performed in matrix-free samples, i.e., by using solutions of d$_3$-AZM (10 mM) and d$_0$-AZM (10 mM) in DMSO. Standard curves were prepared on three different days by derivatizing mixtures containing a fixed amount of d$_3$-AZM (50 nmol; 5 µL) and different amounts of d$_0$-AZM (0 nmol, 0 µL; 40 nmol, 4 µL; 80 nmol, 8 µL; 120 nmol, 12 µL; 160 nmol, 16 µL; 200 nmol, 20 µL) and by analyzing the samples by GC-MS in the SIM mode: m/z 83, m/z 86, m/z 581 and m/z 584 each a dwell-time of 50 ms. Triplicate injections were performed for each concentration point. The concentration of d$_3$-AZM in the toluene phases (each 200 µL) was considered (assuming quantitative derivatization and extraction) to be 250 µM. The concentrations of d$_0$-AZM in the toluene phases (each 200 µL) were considered (assuming quantitative derivatization and extraction) to be 0, 200, 400, 600, 800 and 1000 µM. These concentrations were chosen in order to simulate pharmacologically relevant concentrations for acetazolamide in human urine.
Linear regression analysis between the PAR of \( m/z \) 581 to \( m/z \) 584 (\( y_1 \)) measured on three days and the nominal acetazolamide concentration (\( x_1 \)) yielded the regression equation \( y_1 = 0.0581(\pm 0.016) + 0.0031(\pm 0.00003)x_1 \) (\( r=0.997 \)). Linear regression analysis between the PAR of \( m/z \) 83 to \( m/z \) 86 (\( y_2 \)) measured and the nominal acetazolamide concentration derivatized (\( x_2 \)) yielded the regression equation \( y_2 = 0.1024(\pm 0.019) + 0.0028(\pm 0.00003)x_2 \) (\( r=0.994 \)). The reciprocal slope values of the regression equations provide the concentration of the internal standard \( d_3 \)-AZM. In this experiment, the \( d_3 \)-AZM concentration is calculated to be 323 µM using the PAR of \( m/z \) 581 to \( m/z \) 584, and 357 µM using the PAR of \( m/z \) 83 to \( m/z \) 86. Both values differ from the nominal \( d_3 \)-AZM concentration of 250 µM considering the delivered amount of \( d_3 \)-AZM. The concentration of the internal standard was corrected using the experimentally observed data. Accuracy and precision data obtained using the PAR of \( m/z \) 581 to \( m/z \) 584 (corrected \( d_3 \)-AZM concentration, 323 µM) and the PAR of \( m/z \) 83 to \( m/z \) 86 (corrected \( d_3 \)-AZM concentration, 357 µM) are summarized in Table S1. GC-MS chromatograms from the analysis of acetazolamide (at 600 µM) in DMSO and urine are shown in Figure S4.

Linear regression analysis between the corresponding PAR values of \( m/z \) 83 to \( m/z \) 86 (\( y \)) and the PAR values of \( m/z \) 581 to \( m/z \) 584 (\( x \)) resulted in the regression equation \( y=0.05(\pm 0.02)+0.913(\pm 0.009)x \) (\( r=0.995 \)). The slope value of this regression equation is very close to the ratio of the corrected concentrations of \( d_3 \)-AZM, i.e., 323/357=0.905. In method validation experiments and in quantitative analyses of acetazolamide in urine samples, the above mentioned corrected \( d_3 \)-AZM concentrations were used.

The retention time of the R-SO\(_2\)-N(PFB)\(_2\) derivatives in the standardization experiment described above was determined to be (mean±SD, \( n=124 \)) 8.33±0.008 min (RSD, 0.09%) for \( d_3 \)-AZM and 8.35±0.007 min (RSD, 0.09%) for \( d_0 \)-AZM (paired \( t \) test, \( P<0.0001 \)). The ratio of the retention times of \( d_0 \)-AZM and \( d_3 \)-AZM was calculated to be (mean±SD) 1.002±0.001 (RSD, 0.1%), indicating a highly reproducible GC behaviour of the R-SO\(_2\)-N(PFB)\(_2\) derivatives of AZM. The PFB derivative of \( d_3 \)-AZM eluted constantly in
front of the PFB derivative of d₀-AZM, indicating an interaction of the acetyl groups of the PFB derivatives of d₃-AZM and d₀-AZM with the stationary phase of the GC column.

3.4. Accuracy and precision of the method for urinary acetazolamide

Method validation was performed using urine samples from three healthy volunteers. Native urine samples were used without dilution, yet centrifugation was performed to remove solid material. Aliquots (50 µL) of clear supernatants were used throughout. The internal standard d₃-AZM was added to urine samples at a final added concentration to cover a pharmacologically relevant concentration range (0 – 1000 µM). The method was validated on three days in triplicate for each concentration using the SIM of \( m/z \) 83, \( m/z \) 86, \( m/z \) 581 and \( m/z \) 584. d₀-AZM were calculated by multiplying the PAR of \( m/z \) 581 to \( m/z \) 584 with the d₃-AZM concentration of 323 µM and the PAR of \( m/z \) 83 to \( m/z \) 86 with the d₃-AZM concentration of 357 µM (see above). The results from the validation of the GC-MS method for acetazolamide in the human urine samples from three healthy subjects are summarized collectively in Table 1. In the urine samples spiked with d₃-AZM only, the concentration of d₀-AZM was determined to be around 1 µM, likely originating from unlabeled acetazolamide (d₀-AZM) present in the commercially available d₃-AZM preparation (see above). The PAR values (\( y \)) were linear in the entire concentration range of added AZM (\( x \)). Precision (RSD) ranged between 5.3% and 12.9% for the spiked urine samples. Accuracy (recovery) ranged between 97.8% and 109.1% for AZM added to the urine samples. These data indicate that the GC-MS method is precise and accurate for the measurement of AZM in human urine.

3.5. Limit of detection (LOD) of the method for acetazolamide

For the determination of the LOD value of the GC-MS method, 50 nmol of d₀-AZM and 50 nmol of d₃-AZM (each 5 µL of two separate 10 mM solutions in DMSO) were combined and derivatized as described above. The residue was reconstituted in 200 µL toluene aliquots corresponding to an amount of 250 pmol per 1 µL. Subsequently, serial 1:1-dilutions (by volume) with toluene were performed to construct samples containing
125, 62.5, 31.3, 15.6, 7.8, 3.9 pmol per 1 µL toluene. Each 1 µL of the samples were injected in triplicate and analyzed by SIM of m/z 83, m/z 86, m/z 581 and m/z 584.

The PAR values of m/z 581 to m/z 584 and of m/z 83 to m/z 86 were determined to be (mean±SD, n=21) 0.745±0.011 and 0.797±0.035 ($P<0.0001$, Wilcoxon test), respectively. The ratio of the signal-to-noise (S/N) values of the peak areas of m/z 581 and m/z 584 was determined to be (mean±SD, n=21) 1.33±1.43. The ratio of the S/N values of the peak areas of m/z 83 and m/z 86 was calculated to be (mean±SD, n=21) 1.16±0.57. The S/N values of m/z 581, m/z 584, m/z 83 and m/z 86 correlated with the injected amounts of $d_0$-AZM and $d_3$-AZM derivatives ($P<0.0001$ for all). The corresponding Spearman correlation coefficients were 0.948, 0.916, 0.940 and 0.918.

Linear regression analysis between the S/N values of m/z 581, m/z 584, m/z 83 and m/z 86 ($y$) and the injected amounts of $d_0$-AZM and $d_3$-AZM ($x$, pmol) resulted in the regression equations $y=-395+53x$ ($r=0.944$), $y=601+30x$ ($r=0.633$), $y=68+2.1x$ ($r=0.876$), and $y=83+1.98x$ ($r=0.813$), respectively.

The S/N values of the lowest injected amount (each 3.9 pmol of $d_0$-AZM and $d_3$-AZM) were determined to be 38.7±3.2 (RSD, 8.3%) for m/z 581, 41.0±8.0 (RSD, 19.5%) for m/z 584, 18.3±4.55 (RSD, 24.6%) for m/z 83, and 14.7±6.7 (RSD, 45%) for m/z 86. These data indicate that SIM of m/z 581 and m/z 584 is associated with a higher precision compared to SIM of m/z 83 and m/z 86 for very low AZM concentrations. Extrapolation of the mean S/N value of 39 to an S/N value of 3 (for m/z 581) yields an LOD value of about 0.3 pmol (300 fmol, 67 pg) for the $d_0$-AZM derivative (SIM of m/z 581, m/z 584, m/z 83 and m/z 86; 50 ms dwell-time for each ion).

### 3.6. Limit of quantitation (LOQ) for acetazolamide in human urine

The LOQ of the method for urinary acetazolamide was determined in the same urine samples (each three 50 µL aliquots) used in the method validation. In this experiment, urine samples were spiked with $d_0$-AZM to reach final added concentrations of 0, 25, 50, 100, 150 and 200 µM. The concentration of $d_3$-AZM added to the urine
samples was 250 µM. Each toluene extract was injected three times and SIM of m/z 581, m/z 584, m/z 83 and m/z 86 with 50 ms dwell-time for each ion was performed.

Plotting the PAR of m/z 581 to m/z 584 ($y_1$) versus the concentration of d$_0$-AZM added to urine ($x_1$, µM) yielded the regression equation $y_1=0.018+0.00381x_1$ ($r=0.9983$). Plotting the PAR of m/z 83 to m/z 86 ($y_2$) versus the concentration of d$_0$-AZM added to urine ($x_2$, µM) yielded the regression equation $y_2=0.018+0.0035x_2$ ($r=0.9943$). The reciprocal slope values correspond to d$_3$-AZM concentrations of 262 µM and 283 µM, respectively, which are close to the nominal concentration of 250 µM.

Acetazolamide was determined in the urine samples of this experiment with a precision ranging between 1.9% and 7%, and with a recovery ranging between 101% and 120%. The lowest added d$_0$-AZM concentration of 25 µM was determined with a precision of 5% (using m/z 581 and m/z 584) and 7% (using m/z 83 and m/z 86). The corresponding recovery values were 120.5% and 120%. These data suggest that the LOQ value for acetazolamide in 50 µL aliquots human urine samples is 25 µM or lower using SIM of m/z 581, m/z 584, m/z 83 and m/z 86. The results of this experiment are summarized in Table 2.

3.7. Stability of PFB derivatives of acetazolamide in toluene

The stability of the PFB derivatives of d$_0$-AZM and d$_3$-AZM in toluene extracts from 50 µL aliquots of a human urine sample spiked with 0, 250, 500, 750 and 1000 µM of d$_0$-AZM and with the fixed concentration of 250 µM of d$_3$-AZM (see above) were analyzed immediately after sample work up and after storage at room temperature (about 18 °C) for 21 days.

Linear regression analysis between the PAR values of m/z 581 to m/z 584 ($y_1$) and d$_0$-AZM concentration in urine ($x_1$) resulted in the regression equations $y_1=0.074+0.0036x_1$ ($r=0.9950$) for day 1 and $y_1=-0.006+0.0039x_1$ ($r=0.9969$) for day 21. Linear regression analysis between the PAR values of m/z 83 to m/z 86 ($y_2$) and the d$_0$-AZM concentration in urine ($x_2$) resulted in the regression equations $y_2=0.135+0.0034x_2$ ($r=0.9877$) for day 1 and $y_2=0.011+0.0037x_2$ ($r=0.9965$) for day
21. Considering the data of all samples of this experiment, the peak area (arbitrary unit, a.u.) of the d$_3$-AZM derivative (m/z 584) decreased by a mean factor of 2.6 (mean±SEM; from 7,186,105±71,508 a.u. on day 1 to 2,807,011±98,903 a.u. on day 21). The corresponding values for the peak area of m/z 86 decreased by a mean factor of 1.5 (mean±SEM; from 4,379,094±86,864 a.u. to 3,001,832±183,046 a.u.). The PAR of m/z 86 to m/z 584 was (mean±SEM) 0.586±0.022 on day 1 (PAR$_{1d}$) and 1.096±0.039 on day 21 (PAR$_{21d}$), resulting in a PAR$_{21d}$/PAR$_{1d}$ ratio of 1.94±0.16. These data indicate that the PFB derivatives of d$_0$-AZM (from urine samples spiked with 0 – 1000 µM d$_0$-AZM) and d$_3$-AZM (from the same urine samples spiked at 250 µM) in toluene are relatively stable when stored for 21 days at 18 °C.

3.8. Biomedical application

We tested the usefulness of our GC-MS method in the setting of a pilot study. One healthy volunteer (female, 29 years of age, 70 kg), being author of this article, ingested 250 mg acetazolamide (one tablet Acemit® from medphano, Germany). Before (time zero) and after (up to 5 h) drug administration urine samples were collected in polypropylene bottles pre-cooled in an ice bath. The pH of the urine samples was measured, the urine specimens were aliquoted, and the samples were analysed for nitrate, nitrite and creatinine with previously reported GC-MS methods after acidification by using a 20 vol. % acetic acid solution to remove CO$_2$/NaHCO$_3$/Na$_2$CO$_3$ [2,3]. The urinary concentration of acetazolamide was measured in 50 µL aliquots as described in this article by SIM of m/z 581, m/z 584, m/z 83 and m/z 86. The concentration of the internal standard was 250 µM in all urine samples. Urinary excretion of nitrate, nitrite, creatinine and acetazolamide was corrected for urinary creatinine excretion and the creatinine-corrected excretion rates are expressed as µmol analyte per mmol creatine (µmol/mmol).

After a lag-time of about 0.5 h, the creatinine-corrected excretion of acetazolamide increased almost linearly within the subsequent 1.5 h to reach the value 96 µmol/mmol, which remained almost unchanged for the next 3 h (Fig. 3A). SIM of m/z
581 and m/z 584, and SIM of m/z 83 and m/z 86 resulted in closely correlating acetazolamide concentrations using urine volumes of 50 µL \( (r=0.929) \). Similar results were also obtained by using urine volumes of 40, 30, 20, 10 and 5 µL from this study (Figure S5). The creatinine-corrected excretion rates of nitrite and nitrate increased in parallel to reach their maximum values about 1 h after acetazolamide ingestion (Fig. 3B). The pH value of the urine samples collected in this experiment increased from 7.10 before acetazolamide ingestion to 7.65 after 1.5 h (Fig. 3C). The highest concentration of acetazolamide measured in the urine samples of this experiment was 278 µM. GC-MS chromatograms from analyses of urine samples from this experiment are shown in Figure 4. The acetazolamide concentrations in the urine samples measured in the present study are in agreement with previously reported data using other analytical methods such as HPLC [7,8,29,30], indicating a relatively constant and long-lasting excretion of acetazolamide. The acetazolamide concentrations in blood of humans ingested acetazolamide have also been reported to be relatively constant for many hours upon acetazolamide ingestion at therapeutical doses [31,32].

The results with respect to urinary excretion of nitrite and nitrate collaborate with our previous observations, indicating reversible acetazolamide-dependent inhibition of nitrite and nitrate reabsorption in the kidney [2,3].

4. Discussion

The sulfonamide group \( (R-SO_2-NH_2) \) is common to many classes of drugs. Acetazolamide is one of the oldest and structurally simplest and still therapeutically used sulfonamide drugs, administered for the treatment of many diseases [1], but also used as a potential performance-enhancing drug in sport doping [5]. We have recently shown that acetazolamide ingested at therapeutic doses by healthy subjects enhanced excretion of \( \text{HCO}_3^- \) and inorganic nitrite \( (\text{NO}_2^-) \) in urine [2–4], most likely by inhibiting nitrite-dependent renal CA activity and by additional not yet well understood mechanisms. This newly recognized pharmacological action of acetazolamide may be of particular importance, since \( \text{NO}_2^- \) is considered an abundant reservoir of NO, one of the strongest
endogenous vasodilators. In order to better understand the effects of acetazolamide on nitrite excretion in vivo in humans, reliable analytical methods for the quantitative measurement of acetazolamide in urine are required.

Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is one of the most efficient analytical techniques used to measure numerous analytes in biological samples, commonly with negligible labour and without analyte derivatization. This has been recently demonstrated for the measurement of acetazolamide in human plasma [6]. Historically, GC-MS was the first instrumental technique combining chromatographic with MS separation and gained a wide application area in various disciplines, notably including pharmacology. Concerning acetazolamide and other sulfonamides, derivatization of the amino group is indispensable in GC-based methods. Alkylation (e.g., with iodomethane) and silylation (e.g., with MSTFA) of the amine group of sulfonamides including acetazolamide have been used for their analysis by GC-MS and GC-MS/MS using electron ionization [14,21]. In GC-MS-based methods using negative-ion chemical ionization (NICI), incorporation of fluorine (F) atoms in the analytes by using F-containing derivatization reagents such as PFB-Br, PFB-Boyl-Cl and PFPA, enhance analytical sensitivity by several orders of magnitude. In the present work, we tested these derivatization reagents and the silylation reagent BSTFA for the GC-MS analysis of acetazolamide and other sulfonamides including methazolamide and dorzolamide. For this we used derivatization procedures that have been turned out to be very useful for some analytes including creatinine [24], dimethylamine [26] and amino acids [27].

In the present work, the most useful derivatization reagent for acetazolamide was found to be PFB-Br. PFB-Br is a versatile derivatization reagent [22]. Derivatization reactions with PFB-Br can be performed directly in aqueous solutions including human plasma and urine, as has been demonstrated by us for carbonate [2], nitrite and nitrate [23], and creatinine [24]. Derivatization reactions with PFB-Br can also be performed indirectly in non-aqueous solutions such as in anhydrous acetonitrile in the presence of a base serving as the catalyst. This derivatization approach is most useful for long-chain carboxylic acids including the eicosanoids [28]. In the present study, we found that
acetazolamide can be derivatized with PFB-Br in anhydrous acetonitrile to form a di-PFB derivative \([\text{RSO}_2\text{N(PFB)}_2]\) under very mild derivatization conditions (60 min, 30 °C). For the measurement of acetazolamide in urine, urine-water is completely removed under a stream of nitrogen. The solid residue is then reconstituted in anhydrous acetonitrile. After derivatization complete removal of acetonitrile, remaining base and PFB-Br reagent is performed under a stream of nitrogen gas; the remaining charge-free PFB derivative of acetazolamide is reconstituted in toluene, which is best suited as a solvent for PFB derivatives. We found that methazolamide and dorzolamide can also be converted to their di-PFB derivatives, suggesting the general use of PFB-Br for the derivatization of the sulfonamide group of organic sulfonamides. Using sulfonamides with larger molecular weights, we found that this derivatization reaction is limited by the size of the sulfonamide analyte rather than by the reactivity of the sulfonamide group towards PFB-Br. To our knowledge this is the first work to report on the derivatization of acetazolamide with PFB-Br.

Due to our interest in acetazolamide as a pharmacological potent carbonic anhydrase (CA) inhibitor [1], we developed and validated a straightforward GC-MS method for the precise and accurate quantitative measurement of acetazolamide in 50 µL aliquots of human urine using a commercially available isotopically labelled internal standard, \([\text{acetylo-}^3\text{H}_3]\)acetazolamide (d3-AZM). NICI of the PFB derivative of acetazolamide generates multiple ions, from which some are characteristic for acetazolamide. These anions are \(m/z\) 581, \(m/z\) 83 and \(m/z\) 58. NICI of the PFB derivative of the internal standard \([\text{acetylo-}^2\text{H}_3]\)acetazolamide generates multiple ions including \(m/z\) 584, \(m/z\) 86 and \(m/z\) 58. The mass fragment at \(m/z\) 58 is obtained from the PFB derivatives of both, unlabeled acetazolamide and \([\text{acetylo-}^2\text{H}_3]\)acetazolamide. Therefore, the ion \(m/z\) 58 cannot be used for quantitative measurements using \([\text{acetylo-}^2\text{H}_3]\)acetazolamide as the internal standard. The method validation demonstrated that SIM of \(m/z\) 581, \(m/z\) 584, \(m/z\) 83 and \(m/z\) 86 is specific for acetazolamide and provides closely comparable results in human urine in a pharmacologically relevant concentration range of acetazolamide.
We applied the method to measure acetazolamide in urine samples collected before and for 5 h after ingestion of a 250 mg acetazolamide (1.11 mmol) tablet by a healthy young volunteer. Two hours after ingestion, the creatinine-corrected excretion rate reached a relative constant plateau at the value of 96 µmol acetazolamide per mmol creatinine. This observation is in line with observations from other studies, which used HPLC with ultraviolet absorbance detection after solvent extraction of acetazolamide [5]. A similar pharmacokinetic behavior has been reported for acetazolamide in human plasma by other groups [31,32], suggesting a fairly constant long-term equilibrium between circulating and excretory acetazolamide.

In the urine samples of the present study we also measured the concentration of nitrite and nitrate by GC-MS as PFB derivatives [23] and the pH value of the urine samples. In confirmation of previous findings of our group, we found that the creatinine-corrected excretion rate of nitrite and nitrate reached temporary maximum values. This observation suggests that acetazolamide inhibited the CA-dependent reabsorption of nitrite and nitrate in the kidney [2-4]. In the present study, urinary pH reached its maximum value approximately 1.5 h post-ingestion. Previously, we found that ingested acetazolamide increased the excretion of bicarbonate in the urine with a kinetics resembling that of nitrite and nitrate [2,3]. All these observations suggest that excretion of nitrite and nitrate in the urine partly depends upon the CA activity in the proximal tubule of the kidney. Further, the different kinetics of urinary (and circulating) acetazolamide compared to that of nitrite, nitrate and bicarbonate suggests that acetazolamide inhibits renal CA activity in two phases. First, acetazolamide inhibits acutely and strongly the CA activity 1 to 2 h post-ingestion, resulting in temporary inhibition of the reabsorption of urinary nitrite, nitrate and bicarbonate in the kidney. Consecutively, acetazolamide inhibits weakly but sustainably the activity of CA; inhibition of the CA activity in this phase is presumably not associated with nitrite and nitrate reabsorption, but is apparently associated with inhibition of urinary bicarbonate reabsorption in the kidney.
Table 3 summarizes main characteristics of our GC-MS method in comparison with those of previously reported LC- and GC-based methods for the measurement of acetazolamide in human urine and other biological samples. For the quantification of acetazolamide in human urine by GC-MS we used only 50 µL urine volume, while others used up to 5 mL urine in the GC-MS methods (Table 3). In the vast majority of reported GC-based methods, acetazolamide was analyzed after derivatization mostly using CH₃I. In our GC-MS method we used PFBBr for derivatization of the sulfonamide group. This allows for a highly sensitive measurement of acetazolamide with minimum urine volume and sample work up. The LOQ value of our method may be reduced by increasing the urine volume and by decreasing the toluene volume. For practical reasons we used 200 µL aliquots of toluene for reconstituting the acetazolamide derivative. Reduction of the toluene volume is limited for technical reasons. In our GC-MS system the minimum solvent volume is about 50 µL in commercially available autosampler vials including microinserts. In LC-based methods, acetazolamide is generally analyzed without any derivatization step as they utilize the UV absorbance of acetazolamide. In HPLC methods with UV absorbance detection, endogenous compounds may interfere with the measurement of acetazolamide in human urine, despite the use of extraction steps such as solvent extraction with ethyl acetate [7]. By using an HPLC system previously reported for the analysis of reduced glutathione (GSH) as o-phthaldialdehyde (OPA) derivative [33], we observed co-elution of unknown endogenous compound(s) with acetazolamide which may become important in the lower µM-range (Figure S6). As the extent of such interferences is unpredictable, accurate measurement of low concentrations of acetazolamide in human urine may be seriously compromised.

5. Conclusion

Acetazolamide, an organic sulfonamide drug (RSO₂NH₂) and a potent CA inhibitor, can be derivatized with PFB-Br in anhydrous acetonitrile using an organic base as the catalyst to form RSO₂N(PFB)₂. Derivatization of acetazolamide of urinary origin is
performed under mild conditions (30 °C, 1 h) after evaporation of the urine water to dryness without prior extraction from the urine. In routine, acetazolamide can be analyzed quantitatively in urine (50 µL urine; range, 0 to 1000 µM acetazolamide; 200 µL toluene) by GC-MS in NICI mode using $\text{[acetylo-}^{2}\text{H}_3]\text{acetazolamide}$ as the internal standard (250 µM). Ingestion of a 250 mg acetazolamide containing tablet by a healthy volunteer resulted in constant creatinine-corrected excretion rates of acetazolamide in the urine and in reversible inhibition of the reabsorption of urinary nitrite and nitrate after about 1.5 h. The PFB-Br derivatization procedure is applicable to the measurement of methazolamide, another sulfonamide. The size of the organic moiety of the sulfonamide drug rather than the reactivity of its sulfonamide group towards PFB-Br limits the application of the GC-MS method to other larger sulfonamides.

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**Conflict of interest**

The authors declare no competing financial interest.

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Tables and Figure legends

Table 1. Inter- and intra-day GC-MS validation data for acetazolamide in urine samples of three healthy volunteers (1, 2, 3) using SIM of m/z 581, 584, 83 and 86

Table 2. Results of the experiment performed to determine the LOQ of the GC-MS method for acetazolamide (AZM) in human urine

Table 3. Reported liquid chromatographic (LC) and gas chromatographic (GC) methods for the quantification of acetazolamide in human urine, plasma or serum.
Figure 1. GC-NICI-MS spectrum obtained from a mixture containing each about 250 pmol of unlabeled acetazolamide (d\textsubscript{0}-AZM) and [acetylo-\textsuperscript{2}H\textsubscript{3}]-acetazolamide (d\textsubscript{3}-AZM) as pentafluorobenzyl (PFB) derivatives eluting at about 8.35 min. Inserts indicate the proposed structures for the anions of the PFB derivatives of d\textsubscript{0}-AZM and d\textsubscript{3}-AZM. Mass fragments differing by 3 Da are shown in blue and red. For simplicity the charge of the anions is not indicated.

Figure 2. GC-NICI-MS spectrum obtained from unlabeled methazolamide (about 250 pmol) as pentafluorobenzyl derivative eluting at about 7.8 min. Insets indicate the proposed structures for the PFB derivative of methazolamide and of mass fragments.

Figure 3. Creatinine-corrected urinary excretion of (A) acetazolamide and (B) nitrate and nitrite, and (C) urinary pH before (0 h) and after ingestion of a 250 mg tablet acetazolamide. The arrows indicate the time point of ingestion.

Figure 4. GC-MS chromatograms from the analysis of acetazolamide in urine (50 µL) collected 2 h after ingestion of a 250 mg acetazolamide tablet (Acemit\textsuperscript{®}). SIM of \textit{m/z} 83, 86, 581 and 584 was performed. The concentration of the internal standard was 250 µM. The retention time of 8.53 min is due to the use of a new GC column.
Table 1 Inter- and intra-day GC-MS validation data for acetazolamide in urine samples of three healthy volunteers (1, 2, 3) using SIM of m/z 581, 584, 83 and 86

| Sample | PAR 581/584 | PAR 83/86 |
|--------|-------------|-----------|
|        | Urine 1     | Urine 2   | Urine 3     | Urine 1     | Urine 2   | Urine 3     |
| Day 1  | y=0.094+0.00332x, r²=0.9907 | y=0.066+0.00321x, r²=0.9977 | y=0.0084+0.00341x, r²=0.9952 | y=0.064+0.00313x, r²=0.9916 | y=0.166+0.00276x, r²=0.9773 | y=-0.058+0.00333x, r²=0.9953 |
| Day 2  | y=-0.055+0.00427x, r²=0.9992 | y=0.041+0.00383x, r²=0.9987 | y=-0.018+0.00403x, r²=0.9994 | y=-0.054+0.00401x, r²=0.9966 | y=0.102+0.00346x, r²=0.9887 | y=-0.058+0.00408x, r²=0.9976 |
| Day 3  | y=0.088+0.00365x, r²=0.989 | y=0.060+0.00357x, r²=0.9988 | y=0.074+0.00362x, r²=0.9984 | y=0.125+0.00326x, r²=0.9693 | y=0.167+0.0032x, r²=0.968 | y=0.133+0.00319x, r²=0.9652 |
| All days | 0.049+0.00369x, r²=0.9968 | 0.056+0.00354x, r²=0.9992 | 0.009+0.0038x, r²=0.9999 | 0.051+0.0034x, r²=0.9971 | 0.145+0.0031x, r²=0.9821 | 0.006+0.0035x, r²=0.9959 |
|        | 1/slope =271 µM | 1/slope =283 µM | 1/slope =283 µM | 1/slope =262 µM | 1/slope =293 µM | 1/slope =284 µM |

| AZM (µM) | AZM measured (µM) (mean ± SD, n=9) | Precision (RSD, %) | Recovery (%) | AZM measured (µM) (mean ± SD, n=9) | Precision (RSD, %) | Recovery (%) |
|----------|-------------------------------------|--------------------|--------------|-------------------------------------|--------------------|--------------|
| 0        | 1.11 ± 0.69                         | 62.0               | not applicable | 1.7 ± 0.9                           | 52.0               | not applicable |
| 200      | 206 ± 18                            | 8.8                | 104.2        | 215 ± 22                            | 10.4               | 106.7        |
| 400      | 417 ± 28                            | 6.7                | 104.0        | 426 ± 26                            | 6.1                | 106.1        |
| 600      | 626 ± 33                            | 5.3                | 104.1        | 656 ± 64                            | 9.7                | 109.1        |
| 800      | 803 ± 66                            | 8.2                | 100.2        | 845 ± 104                           | 12.3               | 105.4        |
| 1000     | 1002 ± 103                           | 10.3               | 100.1        | 980 ± 127                           | 12.9               | 97.8         |
Table 2. Results of the experiment performed to determine the LOQ of the GC-MS method for acetazolamide (AZM) in human urine

| Added AZM (µM) | PAR 581/584 | Measured AZM (µM) | Precision (RSD, %) | Recovery (%) | PAR 83/86 | Measured AZM (µM) | Precision (RSD, %) | Recovery (%) |
|----------------|-------------|-------------------|-------------------|-------------|-----------|-------------------|-------------------|-------------|
| 0              | 0.006±0.004 | 1.57±1.05         | 66.9              | not applicable | 0.008±0.002 | 2.26±0.57         | 25.2              | not applicable |
| 25             | 0.121±0.006 | 31.7±1.57         | 5.0               | 120.5       | 0.114±0.008 | 32.3±2.26         | 7.0               | 120         |
| 50             | 0.217±0.007 | 56.9±1.83         | 3.2               | 110.7       | 0.200±0.004 | 56.6±1.13         | 2.0               | 108.7       |
| 100            | 0.396±0.010 | 103.8±2.62        | 2.5               | 102.2       | 0.367±0.019 | 103.9±5.4         | 5.2               | 101.6       |
| 150            | 0.593±0.011 | 155.4±2.88        | 1.9               | 102.6       | 0.550±0.015 | 155.7±4.2         | 2.7               | 102.3       |
| 200            | 0.777±0.019 | 203.6±5.0         | 2.5               | 101.0       | 0.723±0.047 | 204.6±13.3        | 6.5               | 101.2       |
Table 3: Reported liquid chromatographic (LC) and gas chromatographic (GC) methods for the quantification of acetazolamide in human urine, plasma or serum.

| Method          | Matrix          | Column                          | Derivatization | Mobile phase          | Sample preparation                                                                 | LOD; LOQ              | Ref. |
|-----------------|-----------------|---------------------------------|----------------|------------------------|-------------------------------------------------------------------------------------|-----------------------|------|
| Liquid chromatography |                 |                                 |                |                        |                                                                                     |                       |      |
| LC-MS/MS        | Plasma          | Hypurity advance (50 mm × 4.6 mm) | none           | MeCN + 0.1% FA        | 100 µL plasma + IS + FA. SPE. Evaporation to dryness and reconstitution with 500 µL MeCN + 0.1% FA | n.r.; 50 ng/mL        | [6]  |
| HPLC-DAD        | Urine           | HP-Hypersil ODS-Cls (250 mm × 4.0 mm) | none           | A: phosphate buffer B: MeCN | 2 mL urine + 0.5 g sodium phosphate + 0.5 g NaCl. Extraction with 4 mL EA, addition of 5% lead acetate to the organic phase. Centrifugation, evaporation to dryness, reconstitution in 300 µL MeOH; 10 µL injection | 8 ng/mL               | [7]  |
| HPLC-DAD        | Urine           | HP-LiChrospher 100 RP 18 (125 mm × 4.0 mm) | none           | MeCN-H₂O               | native urine; column-switching technique                                              | 10 ng/mL              | [8]  |
| LC-MS/MS        | Plasma (beagle) | Shimadzu VP-ODS C18 (150 mm × 2.0 mm) | none           | A: H₂O; B: MeCN        | 100 µL plasma + IS. Protein precipitation with 600 µL MeCN.                          | n.r.; 200 ng/mL       | [9]  |
| UHPLC-HRMS      | Urine           | Zorbax SB-C8 (2.1 mm × 50 mm)    | none           | A: H₂O + 1 mM ammonium acetate + 0.001% AA B: MeOH + 1 mM ammonium acetate + 0.001% AA | 100 µL urine + IS; centrifugation                                                   | 50 ng/mL; n.r.        | [10] |
| LC-MS/MS        | Urine           | Supelco Discovery HS-C18 (50 mm × 2.1 mm) | none           | A: H₂O + 0.2% FA B: MeOH + 0.2 % FA | SPE. Evaporation to dryness and reconstitution with H₂O + 0.2 % FA. Filtration through a 0.45-μm membrane. | 25 ng/mL; n.r.        | [11] |
| UHPSFC-MS/MS    | Urine           | Acquity UPC2 BEH (100 mm × 3.0 mm) | none           | CO₂ + MeOH + 10 mM FA + 2% H₂O | 100 µL urine and 10-fold dilution with H₂O-MeCN                                      | 0.15 ng/mL; n.r.      | [12] |

Gas chromatography
| Method          | Sample Type | Column Type          | Mobile Phase            | Derivatization | Detection限值 | Reference |
|-----------------|-------------|----------------------|-------------------------|----------------|--------------|-----------|
| GC-ECD         | Serum       | Glass columns (150 x 0.18 cm) | CH₃I | Nitrogen | 100 µL serum + IS + 1 mL tetrapentylammonium + 0.2 mL NaOH + H₂O. Addition of 1 mL CH₂Cl₂ with 5% CH₃I. Evaporation of the organic phase, reconstitution with 1 mL toluene washing with aqueous silver sulphate. | 500 ng/mL | [13] |
| GC-MS          | Urine       | HP Ultra 1 (25 m x 0.20 mm) | CH₃I | Helium   | 1 mL urine + 25 µL NaOH + 6 mL CH₃I + 150 µL tetrahexylammonium hydroxysulphate + 5 mL CH₃I. Extraction with toluene, centrifugation. Filtration through SM-7 resin, evaporation to dryness and reconstitution in 100 µL toluene. | 50 ng/mL; n.r. | [14] |
| GC-MS          | Urine       | HP (25 m x 0.20 mm)      | CH₃I | Helium   | 5 mL urine + IS, filtration through XAD-2 resin. Evaporation to dryness and reconstitution with 200 µL Me₂CO. Addition of 20 µL CH₃I and K₂CO₃. Derivatization for 3 h in 60 °C. | n.r. | [15] |
| GC-MS          | Urine       | HP (5 m x 0.10 mm)       | CH₃I | Helium   | 2 mL urine + 80 µL NaOH + 1 mL CH₂Cl₂-2-propanol. Reconstitution with 50 mg K₂CO₃ + 400 µL of Me₂CO/Me₃I. Derivatization using microwaves for 10 min at 900 W. Evaporation to dryness and reconstitution with 100 µL Me₂CO. | 3 ng/mL | [16] |
| GC-MS          | Urine       | HP (12 m x 0.20 mm)      | CH₃I | Helium   | 2 mL urine + 0.02 THA + 6 mL CH₃I in toluene. Derivatization for 30 min at 50 °C. SPE. Evaporation to dryness and reconstitution with 50 µL EA. | 10 ng/mL; n.r. | [17] |
| GC-MS          | Urine       | HPS (18 m x 0.2 mm)      | CH₃I | Helium   | 5 mL urine + IS. SPE. Evaporation to dryness. Derivatization with CH₃I in acetone + 50 mg K₂CO₃ under microwave irradiation. | 50 ng/mL | [18] |
| GC-MS          | Urine       | Agilent Ultra 1 (17 m x 0.2 mm) | MSTFA NH₄I, PrSH | Helium | 2.5 mL urine + acetate buffer + IS + Na₂CO₃. Extraction with diethylether-2-propanol (5:1, v/v) + Na₂SO₄. Evaporation to dryness. Two-step silylation: 1) 50 µL MSTFA + MeCN (10 min, 80 °C); 2) 50 µL MSTFA/NH₄I/PrSH (10 min, 80 °C) | n.r. | [19] |
| GC-MS          | Urine       | Optima 17 (15 m x 0.25 mm) | PFB-Br | Helium | 50 µL urine + IS, evaporation to dryness. Addition of 100 µL EtOH, evaporation to dryness. Reconstitution with 100 µL MeCN + 10 µL PFB-Br + 10 µL Hüning base, 60 min at 30 °C. Evaporation to dryness and reconstitution with 200 µL toluene. | 0.3 pmol; 25 µM | Present study |

Abbreviations. AA, acetic acid; EA, ethyl acetate; EtOH, ethanol; FA, formic acid/formate; IS, internal standard; MeCN, acetonitrile; MeOH, methanol; Me₂CO, acetone; n.r., not reported; SPE, solid-phase extraction.
Urinary acetazolamide (μmol/mmol) vs. Time after acetazolamide ingestion (h)

- From m/z 581/584
- From m/z 83/86

$r = 0.929$, $P = 0.002$
Highlights

The sulfonamide group of acetazolamide was derivatized for the first time with pentafluorobenzyl bromide.

Other sulfonamides were also derivatized for the first time with pentafluorobenzyl bromide.

Pentafluorobenzyl derivatives of acetazolamide and other sulfonamides are useful for GC-MS.

The validated method was used to quantify pharmacological acetazolamide in human urine.