Drug Metabolism Letters, 2016, *10*, 295-305

RESEARCH ARTICLE

The Metabolism of Methazolamide in Immortalized Human Keratinocytes, HaCaT Cells

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ARTICLEHISTORY

Received: June 03, 2016
Revised: January 12, 2017
Accepted: January 20, 2017

DOI:
10.2174/1872312811666170127160931

Abstract: ***Objective*:** Drug therapy is occasionally accompanied by an idiosyncratic severe toxicity, which occurs very rarely, but can lead to patient mortality. Methazolamide, an anti-glaucomatous agent, could cause severe skin eruptions called Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN). Its precise etiology is still uncertain. In this study, the metabolism of methazolamide was investigated in immortalized human keratinocytes to reveal the possible mechanism which causes SJS/TEN.

***Methods*:** The metabolism of methazolamide was studied using immortalized human keratinocytes, HaCaT cells. HPLC was used to isolate a metabolite from the culture medium. Mass spectrometry (LC-MS/MS) was employed for its characterization. Three typical chemical inducers were assessed for the inducibility of cytochrome P450, and methimazole was used as the inhibitor of flavin-containing monooxygenase (FMO).

***Results*:** A sulfonic acid, *N*-[3-methyl-5-sulfo-1,3,4-thiadiazol-2(3H)-ylidene]acetamide (MSO) was identified as the final metabolite. Dexamethasone and β-naphthoflavone behaved as an inducer of cytochrome P450 in the metabolism, but isoniazid did not. The effect of methimazole was not consistent. We did not detect any glucuronide nor any mercapturic acid (*N*-acetylcysteine conjugate).

***Conclusion*:** *N*-[3-methyl-5-sulfo-1,3,4-thiadiazol-2(3H)-ylidene]acetamide (MSO) is not considered to be a direct product of an enzymatic reaction, but rather an auto-oxidation product of *N*-[3-methyl-5-sulfe-1,3,4-thiadiazol-2(3H)-ylidene]acetamide, a chemically unstable sulfenic acid, which is produced by cytochrome P450 from the β-lyase product of cysteine conjugate of methazolamide. MSO is considered to be susceptible to glutathione and to return to glutathione conjugate of methazolamide, forming a futile cycle. A hypothetical scenario is presented as to the onset of the disease.

Keywords: Cytochrome P450, glutathione, methazolamide, *N*-[3-methyl-5-sulfo-1,3,4-thiadiazo-2(3H)-ylidene]acetamide, Stevens-Johnson syndrome, sulfenic acid, sulfonic acid, toxic epidermal necrolysis.

INTRODUCTION

Drug therapy is occasionally accompanied by severe immune-mediated idiosyncratic toxicity. Except for IgE-mediated immediate-type reactions ranging from urticaria to anaphylactic shock, delayed-type reactions such as severe skin eruptions and interstitial pneumonia are also postulated to be T-cell-mediated immune disease [1, 2]. This type of adverse reaction occurs very rarely, but can lead to patient mortality and occasionally necessitates withdrawal of the drug from market.

Methazolamide (USP 39), a carbonic anhydrase inhibitor, has been used as an antiglaucomatous agent for more than 50 years now. In addition, recent studies have revealed this medication shows interesting diverse effects, and thus much attention has been paid to its other medical potentials: hepatic insulin-sensitizing effect (anticipating therapy for type 2 diabetes [3, 4]) and neuron-protecting effect (anticipating therapy of central nervous system degenerative diseases [5, 6]). However, methazolamide [7-9] has reportedly been involved in the incidence of Stevens-Johnson syndrome (SJS) [10] and toxic epidermal necrolysis (TEN) [11]. Currently the two diseases are understood to be variants of the same process [12, 13]. The symptoms of the diseases are characterized by such clinical manifestations as extraordinary and

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1874-0758/16 $58.00+.00

generalized skin eruptions with continued fever, inflamed buccal mucosa and severe purulent conjunctivitis [10]. The etiology of the diseases is still uncertain, but we hypothesize that the two diseases are not caused by methazolamide itself but by its metabolite(s) [14].

In the preceding report [14], we described the isolation and identification of the metabolites of methazolamide from guinea pig urine. They were a glucuronide, (2-acetylimino-3-methyl-Δ^4-1,3,4-thiadiazol-5-yl)-1-thio-β-D-glucopyranosiduronic acid (referred to as MGU in this text), and a sulfonic acid, *N*-[3-methyl-5-sulfo-1,3,4-thiadiazol-2(3H)-ylidene]acetamide (referred to as MSO). Involvement of β-lyase and cytochrome P450 or flavin-containing monooxygenase (FMO) was suggested. This glucuronide was considered to be resistant to hydrolysis since the linkage of the glucuronic acid moiety and the aglycon was a thioether bond. The sulfonic acid was also considered not to be chemically reactive. This compound is thought to be not a direct product of an enzymatic reaction, but probably an auto-oxidation product of the sulfenic acid, *N*-[3-methyl-5-sulfe-1,3,4-thiadiazol-2(3H)-ylidene]acetamide, which may be produced by oxidation of the sulfur-containing fragment (β-lyase product) or by S-oxidation of the cysteine conjugate [14]. No mercapturic acid (*N*-acetyl cysteine conjugate) was detected in this animal's urine [14].

Sulfenic acids are chemically unstable and reactive [15], and could produce sulfenic acid adduct of cellular macromolecules [16-21] unless reduction by cellular glutathione proceeds rapidly to yield a stable reduced product or subsequent further oxidation reaction(s) occurs immediately to yield a stable oxidation product, *e.g*. sulfonic acid [20, 21]. If keratinocytes produce the sulfonic acid, then we could shed light on the etiology of the disease. Therefore, we studied the metabolism of methazolamide in human keratinocytes, HaCaT cells. HaCaT cells were established in 1988 as a spontaneously transformed cell line from histologically normal skin [22]. Since then, the cells have been employed in various research projects in dermatological area including the toxicology of the area [23-26].

In the present communication, we report that HaCaT cells do not produce glucuronide nor mercapturic acid but sulfonic acid in the metabolism of methazolamide. We postulate that β-lyase and cytochrome P450 are involved in the metabolic process, forming a sulfenic acid as a chemically reactive intermediate. Since the final product, MSO, is thought to be susceptible to glutathione conjugation, probably a futile cycle may be formed to aggravate the production of the intermediate.

MATERIALS AND METHODS

Reagents

Methazolamide, acivicin, isoniazid and methimazole were purchased from Sigma Chemicals (St. Louis, MO, USA). Dexamethasone, β-naphthoflavone, dimethylsulfoxide (DMSO), acetic acid and acetonitrile were obtained from Wako Pure Chemicals (Osaka, Japan). In quantitative studies, we employed purified methazolamide. It was purified in our laboratory by HPLC as described in the next paragraph. Cysteine, cysteinylglycine, and glutathione conjugates of methazolamide were prepared in our laboratory according to the method described in the preceding paper [27]. *N*-[3-Methyl-5-sulfo-1,3,4-thiadiazol-2(3H)-ylidene]acetamide (MSO), and *N*-(3-methyl-5-mercapto-Δ^4-1,3,4-thiadiazol–2-yl)acetamide (MSH) were also synthesized in our laboratory [14]. Fig. (**1**) shows the chemical structure of these compounds with their code names as used in this text. Phosphate-buffered saline (PBS) and Dulbecco's MEM were obtained from Nikken Bioscience (Kyoto, Japan). Fetal bovine serum (Cat #12-10378) was supplied by IRH Biosciences (Lenexa, KS, USA).

Pufification of Methazolamide by HPLC

The purchased methazolamide was purified by HPLC using a Hitachi L6200 pump (Tokyo, Japan) equipped with a Shimadzu LPD-6A UV detector (Kyoto, Japan). The stationary phase was a C_{18} column (TSK-Gel, ODS-80TM, 4.6 mm i.d. × 250 mm long, particle size, 5 mm; Tosoh, Tokyo, Japan), and the mobile phase was 0.1% acetic acid:acetonitrile (80:20 (v/v)). Methazolamide appeared at the retention time of 16 min. This fraction was collected, and lyophilized to remove acetic acid. The lyophilized metazolamide was dissolved in a minimum amount of purified water, and again lyophilized. This was repeated one more time. Then the sample was dried at 70^{o}C under reduced pressure for 6 hours.

Cell Culture

HaCaT [22] cells were supplied from Department of Biochemistry, Akita University Graduate School of Medicine. They were cultured in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal bovine serum in 75 cm^2 tissue culture flask (Cat #35-3014, BD-Falcon, Franklin, NJ, U.S.A.) at 37^{O}C under humidified atmosphere (5% CO_2 and 95% air). In sub-confluent condition, the cells were detached with 0.02% EDTA in phosphate buffered saline (PBS), and served for the following experiments.

(1) Detection of Metabolites and the Effect of Acivicin

The detached cells were poured into a 6-well culture plate (#35-3046, BD-Falcon) with a population of 2×10^5 cells in each of the wells (the medium volume was 5 ml), and incubated for 24 hours in the same manner as above to let them adhere to the plate bottom. Then the culture medium was exchanged for the same volume of fresh medium. After another 48 hours, the medium was changed to a fresh one containing 1 mM methazolamide and 0-100 μM acivicin, a specific inhibitor of γ-glutamyltranspeptidase [28, 29]. The culture continued further for 72 hours. Then 0.5 ml of the cultured medium was collected from each well of the plate, and served for HPLC analysis. The rest of the cultured medium was used for mass spectrometry.

(2) The Effect of Cytochrome P450 Inducers

In this study, DMSO was employed as a solubilizing agent for cytochrome P450 inducers. The inducers were first dissolved in DMSO, and then diluted with the culture medium so that the final concentration of DMSO was 0.5% (v/v). The concentration of inducers was in accordance with the literature [30-32].

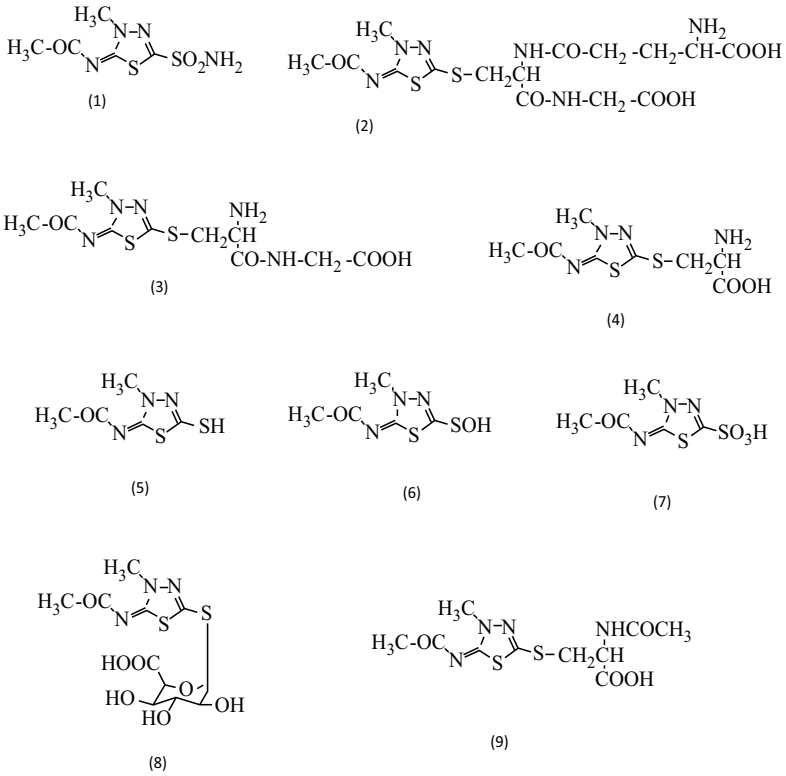

Fig. (1). Chemical structures.
(1) Methazolamide (MZA); (2) Glutathione conjugate of methazolamide (MSG); (3) Cysteinylglycine conjugate of methazolamide (MCG); (4) Cysteine conjugate of methazolamide (MCY); (5) *N*-[3-methyl-5-mercapto-1,3,4-thiadiazo-2-yl]acetamide (MSH); (6) *N*-[3-methyl-5-sulfe-1,3,4-thiadiazol-2(3H)-ylidene]acetamide; (7) *N*-[3-methyl-5-sulfo-1,3,4-thiadiazol-2(3H)-ylidene]acetamide (MSO); (8) (2-acetylimino-3-methyl-D^4-1,3,4-thiadiazol-5-yl)-1-thio-β-D-glucopyranosiduronic acid (MGU); (9) Acetylcysteine conjugate of methazolamide (MAC).
Code names used in the text are indicated in parentheses.

The detached cells were poured into a 6-well culture plate with a population of 2×10^5 cells in each of the wells containing 4 ml of the medium. After the incubation of the cells for the first 24 hours to let the cells adhere to the well bottom, the culture medium of each well was changed with fresh medium containing each of the inducers, and incubated for another 48 hours (pretreatment). Then the well was washed with the same volume of fresh medium containing 1 mM purified methazolamide, 50 μM acivicin and one of the inducers. After that, the culture continued for another 72 hours in the same medium excepting inducer. Then 50 μl of the cultured medium was collected from each well of the plate and applied to the HPLC. In the control experiment, the medium containing only 0.5% (v/v) DMSO was employed. All of the cultures were carried out in sextuplicate.

(3) The Effect of Methimazole

To suppress the activity of flavin-containing monooxygenase, methimazole was used as a competitive inhibitor of the enzyme [33, 34] The study was carried out in the same manner as above. The concentrations of methazolamide and acivicin were 1 mM and 50 μM, respectively. Those of methimazole were 0-500 μM. This was carried out in dodecuplicate.

HPLC Analysis

The collected medium was filtered through an Amicon ultra-0.5mL 3K (Millipore, Billerica, MA, USA) or a Nanosep 3K omega (Pall, Port Washington, NY, USA) to remove high molecular weight components. Fifty to 200 μl of the filtered medium was applied to HPLC. HPLC analysis was carried out using the Hitachi L6200 pump equipped with the Shimadzu LPD-6A UV detector. The stationary phase was the C_{18} column (TSK-Gel, ODS-80TM, 4.6 mm i.d. × 250 mm long, particle size, 5 mm). Unless otherwise described, 50 mM NaH_2PO_4 containing acetonitrile was used as the mobile phase. Acetonitrile concentration was changed as follows: 10% isocratic for 0-10 min, then 10% to 30% gradient for 10-15 min, after that 30% isocratic for 15-29 min. Column temperature was maintained at 25^{O}C. Flow rate was 0.8 ml/min. The UV absorbance at 300 nm was employed for detection. The UV absorbance data was recorded by a data recorder (Shimadzu Chromatopac C-R6A, Kyoto, Japan) as well as by an NEC PC9821 computer through an Ozac A/D converter (Ozac Engineering, Uji, Kyoto, Japan) [35]. The former device was used for quantification of the eluents, and the latter for confirmation of the retention time of the peaks.

Chemically synthesized cysteine, cysteinylglycine, and glutathione conjugates of methazolamide (MCY, MCG and MSG), *N*-[3-methyl-5-sulfo-1,3,4-thiadiazol- 2(3H)-ylidene] acetamide (MSO), (2-acetylimino-3-methyl-Δ^4-1,3,4-thiadiazol-5-yl)-1-thio-β-D-glucopyranosiduronic acid (MGU) and its aglycon, *N*-(3-methyl-5-mercapto-Δ^4-1,3,4-thiadiazol-2-yl)acetamide (MSH), were also subjected to determinations of their retention time for the reference identification.

In the quantification, we employed a serum-derived peak at 10.5 min as the reference. The amount of metabolite was at first obtained as a peak area ratio to that of the serum-derived peak. Then the ratio was compared with that of standard solution for the calculation of the amount excreted from the cells. Since we dealt with relatively small peaks and we did not clean up the samples before injection, we encountered a problem of interference of neighboring peaks. In order to cope with this problem, we also obtained a peak height ratio, and then calculated the ratio, "peak height/peak area". This ratio, "peak height/peak area", was used as a parameter to verify the quantification. We only dealt with the quantification data whose "peak height/peak area" ratio fell between 1.4–1.6. We considered a peak having the ratio, "peak height/peak area", falling outside this range, interfered with neighboring peak(s), and excluded it in estimation. By this criterion, we succeeded in obtaining consistency.

Identification of Metabolites by Mass Spectrometry (LC-MS/MS Study)

In order to confirm the chemical structure of metabolites, we attempted an LC-MS/MS study. A total of 4 ml of the culture medium from the incubation with 1 mM methazolamide and 50 μM acivicin was subjected to HPLC to obtain the fractions A (retention time 7-8 min) and B (retention time 9.5-10.5 min). Each time 500 μl of the medium was injected, and the fractions were collected and combined. The combined solutions were lyophilized to remove the solvent. Then the lyophilized samples were again dissolved in minimal amount of the sublimable solvent, 50 mM ammonium acetate buffer (pH 5.0): acetonitrile (90:10, v/v), and again subjected to HPLC to collect the fractions, and lyophilized. This time, we used this sublimable solvent as the mobile phase.

Liquid chromatography/mass spectrometry (LC-MS/MS) analysis was performed on an Alliance 2695 HPLC separation module/tandem quadrupole MS TQD system (Waters, Milford, MA, USA). Chromatographic separation was achieved on a C_{18} column (TSK-Gel ODS-80TS, 2.0 mm i.d.×150 mm long, particle size, 5 mm; Tosoh, Tokyo, Japan, referred to as the short column in this text). The mobile phase was 0.1% acetic acid:acetonitrile (90:10, v/v). The flow rate was 0.2 ml/min in the isocratic mode. Total run time was 15 min. A tandem quadrupole MS TQD, operated in product ion monitoring and positive electrospray ionization mode, was used for detection. MassLynx 4.1 software was used for data acquisition and processing. MS/MS parameters (precursor ion, product ion, cone energy, and collision energy) of the reference compound, MSO, were optimized by QuanOptimize software. UV absorbance was also monitored at the range of 210-500 nm.

Statistics

Welch's one-tailed t-test was employed to evaluate statistical significance in the difference between two independent groups. This was carried out using a Microsoft Excel function.

RESULTS

The Growth and the Morphology of HaCaT Cells

HaCaT cells grew in a monolayer. Through the whole set of experiments the cells reached to the almost confluent condition. The morphology of the cells was observed under a phase contrast microscope and no differences were observed among the cells used in this study.

Detection of Metabolites by HPLC

The retention time of chemically synthesized compounds were as follows: MSG, 12.0 min; MSH, 7.7 min; MGU, 8.1 min; MCG, 9.2 min (delayed to 9.9 min in the presence of a large peak of MCY); MCY, 9.1 min; MSO, 9.0 min (delayed to 10.1 min in the presence of a large peak of MCY); and MAC, 19.4 min.

HaCaT cells were cultured in the presence of 1 mM methazolamide. Fig. (**2**) is a typical example of chromatograms of cell-cultured media. On the chromatogram, MCY was detected at the retention time of 8.9 min with MCG and MSG at 10 min and 11.7 min, respectively. The peak of MCG accompanied a small shoulder at 10.1 min which agreed with that of MSO. Then we let the cells metabolize

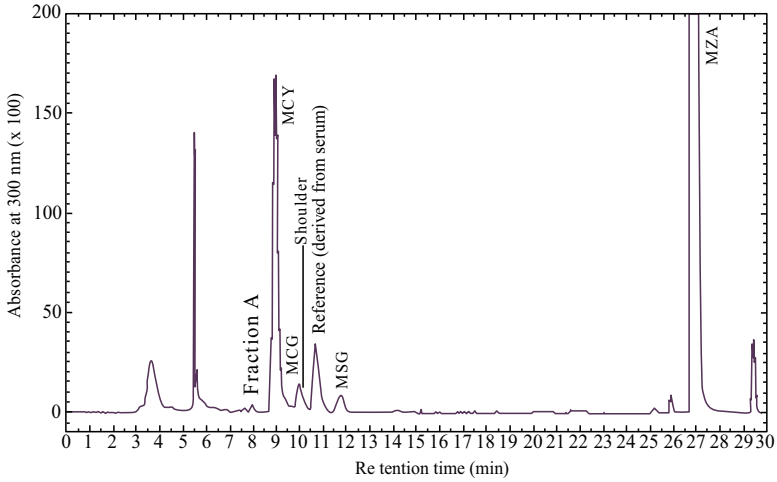

Fig. (2). HPLC analysis.

The culture medium after 72 hours of incubation of HaCaT cells with 1mM methazolamide was analyzed with HPLC as detected by the absorbance at 300 nm. The medium was deproteinized by filtration with Amicon Ultra 3K. Fifty ml of the filtrate was applied to the HPLC.

methazolamide in the presence of 50-100 μM acivicin, an inhibitor of γ-glutamyltranspeptidase [28, 29]. It reduced the peak height of MCY and MCG, which visualized the peak at 10.1 min (Fig. **3**). We collected this peak as the fraction B and lyophilized for LC-MS/MS study to reveal its chemical structure.

The glucuronide (MGU) is expected to appear on the chromatogram at around 8 min. We detected a small peak at 8 min (Fig. **2**), but its UV spectrum showed λ_{max} at 320 nm which did not agree with that of MGU (λ_{max} at 297 nm). We collected this peak as the Fraction A and lyophilized for LC-MS/MS study to reveal its chemical structure.

The *N*-acetylcysteine conjugate (mercapturic acid, MAC) is expected to appear at 19 min on the chromatogram, However, we observed no peak showing absorbance at 300 nm at around 19 min (Fig. **2**).

Confirmation of Metabolites by LC-MS/MS Study

1). Reference Compounds

The retention times of the three reference compounds, MSO, MGU and MSH, on the short column using 0.1% acetic acid:acetonitrile (90:10, v/v) as the mobile phase were 5.9 min, 6.8 min and 7.8 min, respectively. The optimized parameters of MS/MS conditions were as follows: Cone energy was 25 V, and collision energy was 15 V, for the three compounds. In the case of MSO, CID spectrum of the ion at m/z 238 (molecular ion, $[M+H]^+$) showed daughter ions at m/z 195.9 (base peak, M-CH_3CO), m/z 57.0 (relative intensity to the base peak, 16%) and m/z 99.0 (relative intensity to the base peak, 3%). In the case of MGU, CID spectrum of the ion at m/z 366 (molecular ion, $[M+H]^+$) showed daughter ions at m/z 189.9 (base peak, [M-glucuronate]$^+$), m/z 147.9 (relative intensity to the base peak, 19%). MSH showed a molecular ion, $[M+H]^+$ at m/z 190.1 Its CID spectrum showed daughter ions at m/z 147.9 (base peak, [M-CH_3CO+H]$^+$), and at m/z 189.9 (relative intensity to the base peak, 6%).

2). Characterization of the Fraction A

Selected ion recordings at m/z 190.1 ($[M+H]^+$ of MSH, [M-glucuronate]$^+$ of MGU) and m/z 366.2 ($[M+H]^+$ of MGU) detected a UV-absorbing peak at the retention time of 6.6 min. However, the CID spectrum of the ion at m/z 190.1 showed daughter ions at m/z 103.0 (base peak), m/z107.0 (relative intensity to the base peak, 80%), m/z 130.9 (relative intensity to the base peak, 80%), and m/z 143.9 (relative intensity to the base peak, 50%), which did not agree with that of MGU nor MSH. We failed to detect either MGU or its aglycon, MSH, in this fraction A.

3). Characterization of the Fraction B

We detected a UV-absorbing peak at the retention time of 5.2-6 min (the upper panel of Fig. (**4**). The UV absorbance up to 3 min is probably contributed to by the change in diffraction caused by salt elution and/or some other factors. The total ion chromatogram of the daughter ions derived from the parent ion, m/z 238, is shown in the lower panel of Fig. (**4**). The highest signal was observed at 5.9 min. This coincides with the retention time of the synthesized MSO. Fig. (**5A**) is the mass spectrum (CID spectrum of the ion m/z 238) at 5.9 min. The spectrum can be considered to agree with that of the reference compound, MSO. A fragment ion m/z 137 was recorded in this sample but not in the reference (Fig. **5B**). However, this fragment ion also can be thought to be derived from MSO (Fig. **6**).

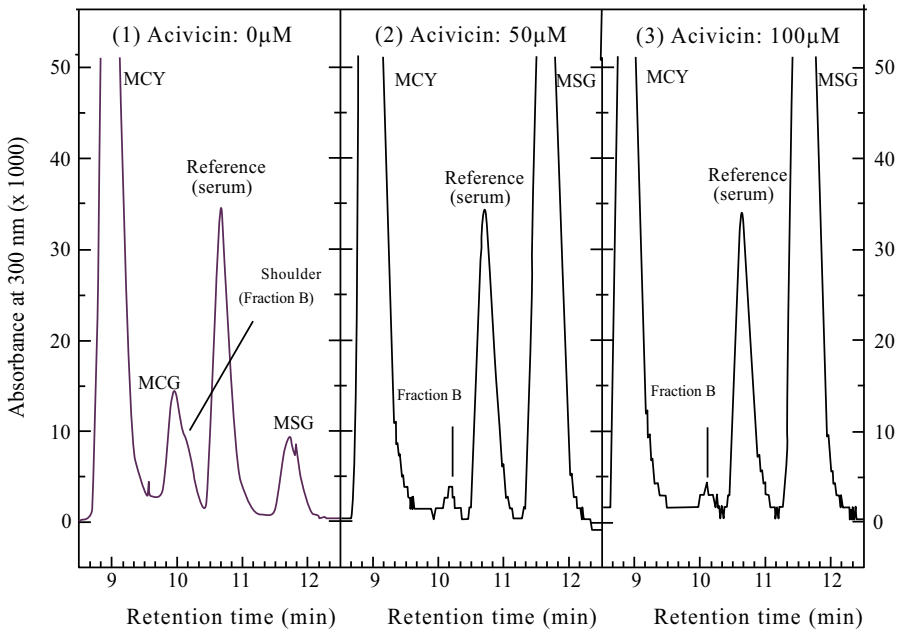

Fig. (3). Chromatogram of the culture medium.

The culture medium after 72 hours of incubation of HaCaT cells with 1 mM methazolamide in the presence of 0-100 μM acivicin was analyzed by HPLC as detected by the absorbance at 300 nm. The graph shows only the data between 8.5 and 12.5 min. Acivicin decreased the production of MCG and therefore lowered the peak height of MCG, which visualized production of MSO. (1) In the absence of acivicin. (2) In the presence of 50 μM of acivicin. (3) In the presence of 100 μM acivicin.

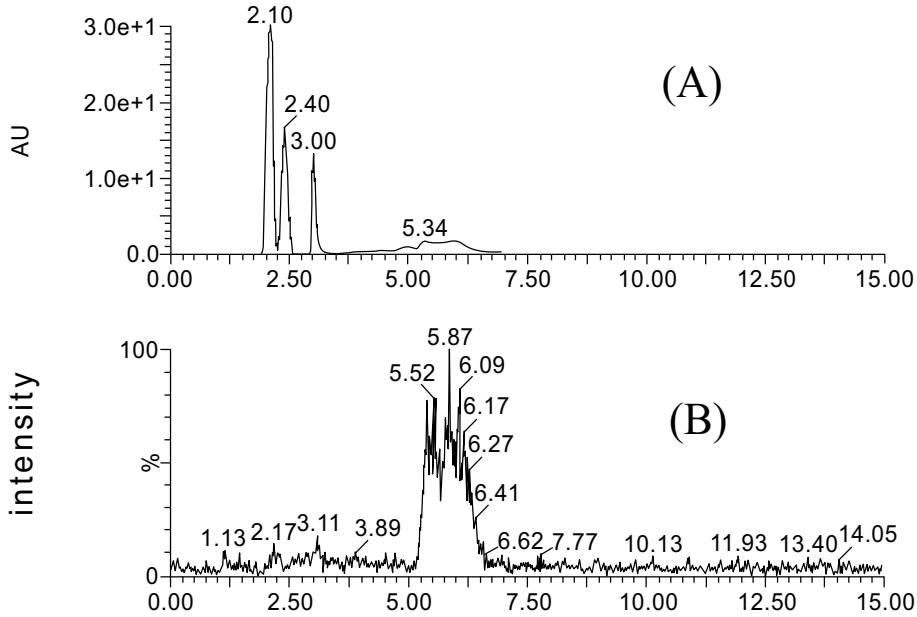

Fig. (4). UV and total ion chromatogram of the fraction B.
Fraction B was concentrated and desalted, and applied to LC-MS/MS study. (**A**) Photodiode array detector recorded UV absorbance between 210 and 500 nm. (**B**) Total ion chromatogram is depicted for the daughter ions of the parent ion m/z 238.

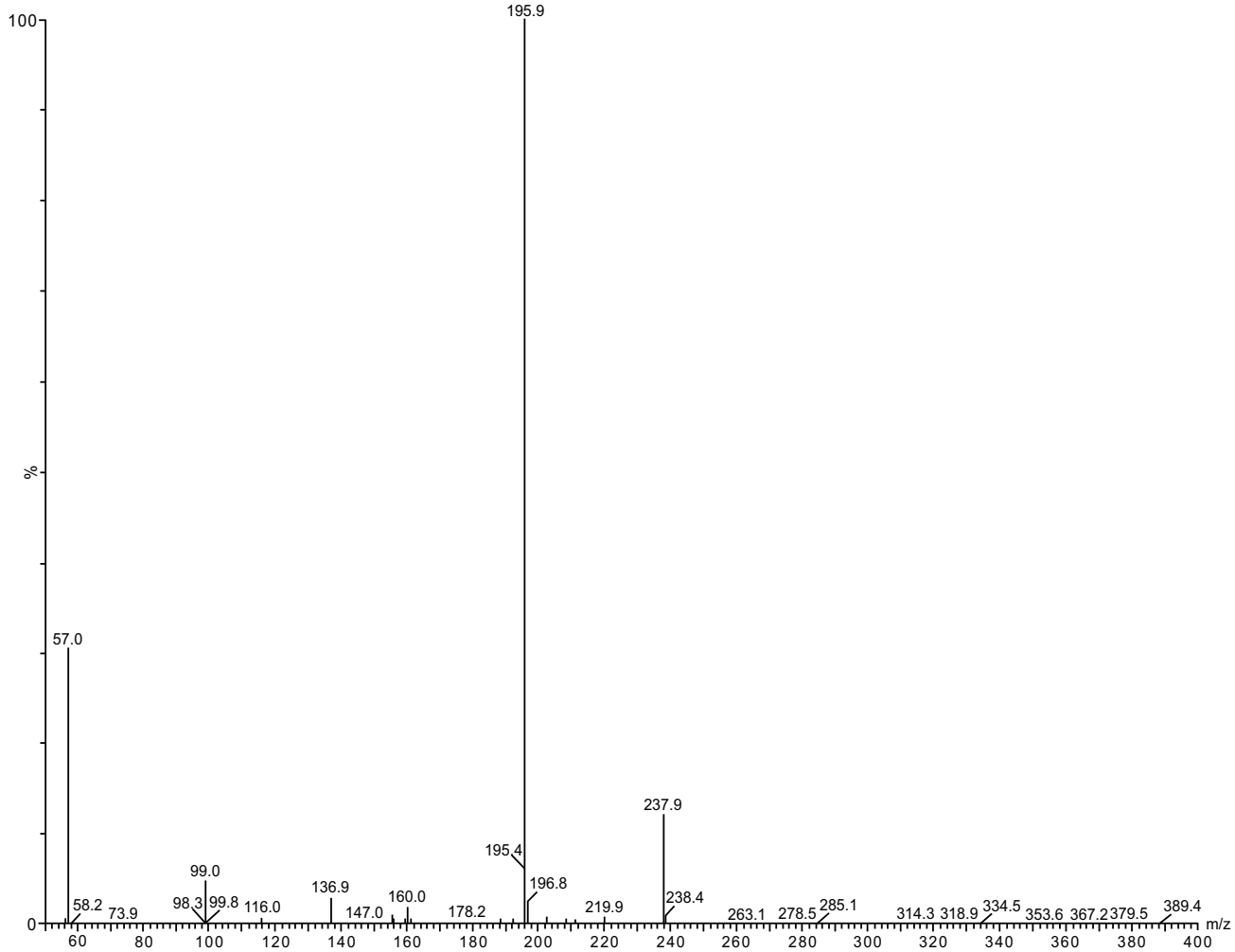

Fig. (5A). CID spectrum of the Fraction B.
CID spectrum of the molecular ion at m/z 238 was recorded. It shows daughter ions at m/z 195.9. m/z 136.9, m/z 99 and m/z 57.

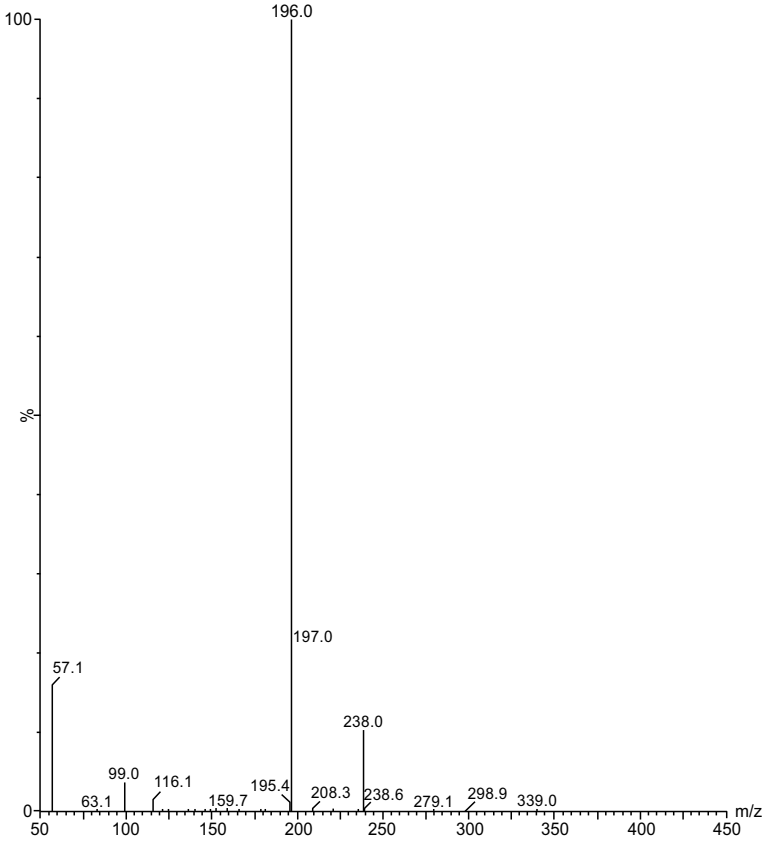

Fig. (5B). CID spectrum of MSO.
CID spectrum of the molecular ion at m/z 238 was recorded. The fraction B shows a similar spectrum (Fig. **5A**) to this one.

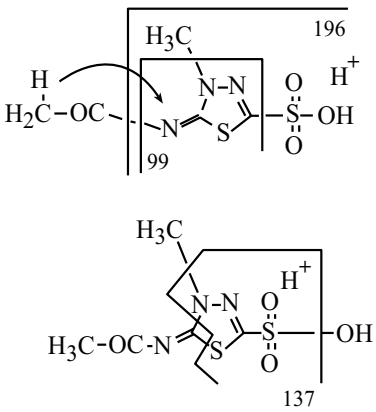

Fig. (6). The structure and the fragmentation of the compound found in fraction B.

Effect of the Inducers of Cytochrome P450

We observed the effect of some chemical inducers of cytochrome P450. Dexamethasone, β-naphthoflavone [30] and isoniazid [31, 32] were attempted. Dexamethasone, and β-naphthoflavone showed an inducing effect but isoniazid did not, as shown by Welch's one-tailed t-test (Table **1**).

Effect of Methimazole

In order to determine if flavin-containing monooxygenase is also playing a role in the metabolism of methazolamide in addition to cytochrome P450, we examined the effect of methimazole on the metabolism. Methimazole lowered MSO excretion, but the effect was not significant throughout the concentration range (Table **2**).

DISCUSSION

In the preceding communication, we presented a plausible metabolic pathway of methazolamide in the guinea pig where we detected MGU and MSO in its urine. In the rat urine we detected only MAC (unpublished observation). In the present study using human keratinocytes, HaCaT cells, we confirmed only MSO as the metabolite of methazolamide. Instead of MGU and its aglycon, MSH, we detected very small amounts of an undefined substance. We are not certain if this is derived from methazolamide. Further study is required to resolve this problem.

The fact that MGU and MAC were not detected but MSO was is very suggestive in the discussion of the etiology of SJS/TEN caused by methazolamide. The former two products are considered to be chemically stable, and therefore not very toxic. Cytochrome P450 is not involved in the formation of these two compounds. MSO itself is also thought not to be toxic. However, MSO is hardly considered to be a direct product of enzymatic reaction, but an auto-oxidation product of *N*-[3-methyl-5-sulfe-1,3,4-thiadiazol-2(3H)-ylidene]acetamide [14], a sulfenic acid.

Sulfenic acid is chemically unstable [15] and thus easily reacts with cellular macromolecules, which may cause

Table 1. Effect of chemical indusers of cytochrome P450.

In Exp 1, cells were incubated for 48 hours without any inducer of P450 after 24 hours following seeding, and then 1 mM methazolamide metabolized for 72 hours in the presence of 50 μM acivicin. After that, the amounts of MSO excreted were determined .

In Exps 2, 3 and 4, the cells were incubated for 48 hours with 50 μM of dexamethasone, isoniazid or β-naphthoflavon, respectively. After that, they were washed with the medium containing 1 mM methazolamide and 50 μM acivicin. Then 1 mM purified methazolamide was metabolized for 72 hours in the presence of 50 μM acivicin and the absence of any inducer. After that, the amounts of MSO excreted were determined.

Welch's one-tailed t-test was done and p value against Exp 1 was culculated.

	Inducers of Cytochrome P450	Amount of MSO Excreted Extracellularly (nmole/4 ml)*	Significance Level (p Value of Welch's T-Test Against Exp 1)
Exp 1	-	1.46±0.40 (6)	-
Exp 2	Dexamethasone	2.86±0.72 (6)	0.002
Exp 3	Isoniazid	1.82±0.37 (6)	0.067
Exp 4	β-Naphthoflavone	2.22±0.54 (6)	0.010

*Mean ± standard deviation with the number of experiments in parenthesis.

Table 2. Effect of methimazole on the metabolism of methazolamide.

In Exp 1, cells were incubated for 48 hours without methimazole after 24 hours following seeding, and then 1 mM methazolamide metabolized for 72 hours in the presence of 50 μM acivicin. After that, the amounts of MSO excreted were determined .

In Exps 2, 3, 4 and 5, the cells were treated in the same manner as Exp 1 for the first 48 hours. Then 1 mM methazolamide was metabolized for 72 hours in the presence of 50 μM acivicin with methimazole at a concentration of 50, 100, 250 and 500 μM, respectively. After that, the amount of MSO excreted were determined.

Welch's one-tailed t-test was done and p values against Exp 1 were calculated, respectively.

	Methimazole Concentration (μM)	Amount of MSO Excreted Ectracellulary (nmole/4 ml)*	Significance Level (p Value of Welch's T-Test Against Exp 1)
Exp 1	0	2.25±0.96 (12)	-
Exp 2	50	1.81±0.26 (12)	0.070
Exp 3	100	1.56±0.392 (12)	0.027
Exp 4	250	1.67±0.398 (12)	0.038
Exp 5	500	1.85±0.358 (12)	0.099

Mean ± standard deviation with the number of experiments in parenthesis.

haptenization of intracellular macromolecules and/or cellular necrosis, leading to immunological reaction in a worst case scenario. The sulfenic acid can be derived from MSH, *N*-[3-methyl-5-mercapto-Δ^4-1,3,4-thiadiazo-2-yl]acetamide, which is a β-lyase product of the cysteine conjugate of methazolamide [36]. The concept that oxidation of thiol group attached to alicyclic and heterocyclic compounds yielding a sulfenic acid is catalyzed by cytochrome P450 or flavin-containing monooxygenase is already established [37, 38].

In the present study, we attempted three cytochrome P450 inducers [30-32] to examine their effect on MSO production in HaCaT cells. Among them dexamethasone and β-naphthoflavone showed an inducing effect. The effect was not very strong, ca. 1.5-2-fold (Table **1**). This may be interpreted as follows: the C-S bond connecting the thiadiazoline ring and the substituent is polarized. The C-atom of the ring is electron deficient due to the electron-withdrawing force of the substituent, and dissociated glutathione attacks it to form a glutathione conjugate [39]. The electron-withdrawing force of $-SO_3H$ is thought to be comparable to that of $-SO_2NH_2$. Thus, it is reasonable to assume that MSO is also susceptible to glutathione conjugation. Therefore, there is a possibility that a certain portion of MSO formed reverts to the glutathione conjugate, MSG, forming a futile cycle. So, the amount of the sulfenic acid formed in cells is thought to be much more than that of MSO. This also means that cytochrome P450 is induced in excess. This should be dependent upon the cellular level of glutathione. This indicates that cellular level of glutathione could work as a toxic agent as well as detoxifying agent.

Dexamethasone is enlisted as an inducer of CYP2A6 and 3A4 [30]. The human CYP3A subfamily plays a dominant role in the oxidation of 40-50% of all currently prescribed drugs [40]. Enhancement of the toxicity of methazolamide

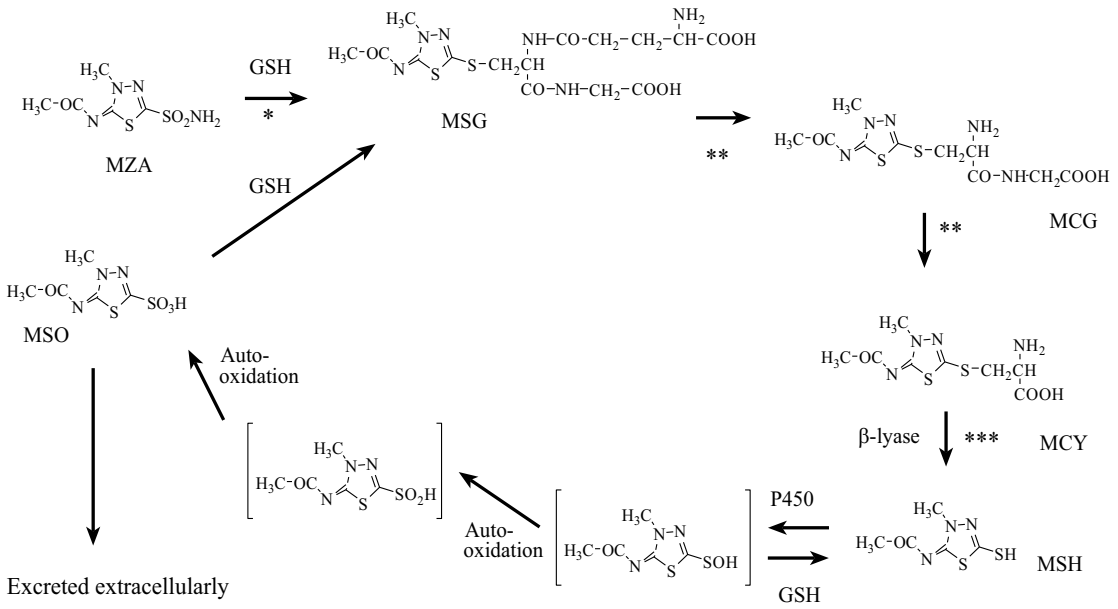

Fig. (7). The metabolism of methazolamide.
The compounds in the square blackets are not yet isolated, and, therefore, speculative. P450 is cytochrome P450. GSH is glutathione (reduced form).
*Conroy, *et al*. [39]; **Kishida, *et al*. [27] ; ***Kishida, *et al*. [42].

by co-administering medications is suspected, but no description is found in their case reports [7-9] from this point of view.

Isoniazid did not behave as an inducer, indicating that CYP2E1 did not participate in the metabolism. This suggests alcoholism did not contribute to the incidence of SJS/TEN caused by methazolamide.

Flavin-containing monooxygenase (FMO) is another enzyme to oxygenate xenobiotics, especially nucleophilic heteroatom compounds [41]. In contrast to cytochrome P450, it is not easily induced nor readily inhibited [41]. Methimazole is a good substrate of the S-oxygenation activity of this enzyme [33], and we, therefore, attempted to employ it as a competitive inhibitor in the metabolism of methazolamide. As can be seen in Table **2**, methimazole did not consistently affect MSO production. In addition, the effect was relatively small. At the present time, we prefer waiving estimation as to FMO's participation in the metabolism of methazolamide. Further study is required.

Considering the above discussion together with the findings in the previous works [14, 36, 42], we propose the whole metabolism of methazolamide in HaCaT cells in Fig. (**7**).

SJS/TEN is now considered to be an autoimmune disease and some studies showed the strong correlation with methazolamide–induced SJS/TEN and HLA-B59 serotype that is often seen in East Asians [9, 43-45]. On the other hand, P450 is reported to the major target of the patients of autoimmune hepatitis, some of which related with certain HLA serotypes [46].

Our present study has revealed involvement of cytochrome P450 in the metabolism of methazolamide. In our early study [42], ^{35}S-labeled cysteine conjugate of methazolamide was incubated with bovine kidney and conjunctiva homogenates. A significant amount of radioactivity was detected from the protein precipitates of both homogenates as bound residues, showing that a metabolite covalently bound to intracellular proteins. In the soluble fraction we detected a metabolite yielding an ion at m/z 190 in mass spectrometry. Later the ion was identified to be that derived from the β-lyase product of cysteine conjugate of methazolamide [36]. These early findings coincide with the present data, which leads us to the following hypothetical scenario on the pathogenesis of SJS/TEN caused by methazolamide. First, cytochrome P450 in the oxidation of the β-lyase product (MSH) yields a sulfenic acid which binds to various intracellular macromolecules and disturbs their function. This leads to tissue necrosis as well as haptenation of intracellular macromolecules, which could trigger metabolite-related immunological reactions [47]. Second, the futile cycle could induce cytochrome P450 in excess. This could contribute to increased vesicular transport of cytochrome P450 via Golgi apparatus, which may lead to expression of cytochrome P450 on cellular membrane [48-50]. This could trigger an autoimmune reaction to cytochrome P450 [1].

In order to demonstrate the precise etiology of methazolamide-induced SJS/TEN, further study is required from the viewpoint of immunology.

CONCLUSION

HaCaT cells metabolized methazolamide, and MSO was isolated as a final metabolite from the culture medium. Neither MGU nor MAC was detected. MSO is hardly considered to be a direct product of an enzymatic reaction, but rather an auto-oxidation product of *N*-[3-methyl-5-sulfe-1,3,4-thiadiazol-2(3H)-ylidene]acetamide, a chemically unstable sulfenic acid, which is produced by cytochrome P450 from β-lyase product of cysteine conjugate of methazolamide. MSO is considered to be susceptible to glutathione

conjugation and to revert to glutathione conjugate of methazolamide, forming a futile cycle. A hypothetical scenario is presented as to the onset of the disease.

LIST OF ABBREVIATIONS

C_{18}	=	Octadecyl
CID	=	Collision-induced decomposition
CYP	=	Cytochrome P450
FMO	=	Flavin-containing monooxygenase
HPLC	=	High performance liquid chromatography
LC-MS	=	Liquid chromatography/mass spectrometry
MS	=	Mass spectrometry
PBS	=	Phosphate-buffered saline
SJS	=	Stevens-Johnson syndrome
TEN	=	Toxic epidermal necrolysis
USP	=	United States Pharmacopea

AUTHORS' PARTICIPATION

T. Sasabe participated in the design of the research, and carried out cell culture studies. SM and YM worked in the area of mass spectrometry studies, and performed to elucidate the chemical structure of an isolated metabolite. KK and MY conducted the biochemical study and carried out HPLC studies in isolation of a metabolite and assessment of chemical inducers of the metabolism. T. Sugiyama conceived the study and supplied the cells. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. K. Nishioka, former professor at the University of Texas M. D. Anderson Cancer Center, Houston, Texas, for reviewing this manuscript.

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