Detection of 6-(Methylsulfinyl)hexyl Isothiocyanate (6-MITC) and Its Conjugate with *N*-Acetyl-L-cysteine (NAC) by High Performance Liquid Chromatograpy-Atmospheric Pressure Chemical Ionization Mass Spectrometry (HPLC-MS/APCI)

Hideaki YAMAGUCHI,*[,a] Toshiro NOSHITA,[a] Yumi KIDACHI,[a] Hironori UMETSU,[b] Yoko FUKE,[c] and Kazuo RYOYAMA[a]

[a] *Department of Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Aomori University; Aomori 030–0943, Japan:* [b] *Department of Life Sciences, Junior Collage, Gifu Shotoku Gakuen University; Nakauzura, Gifu 055–8288, Japan: and* [c] *Department of Food Science and Human Nutrition, Tokyo Metropolitan University; Akishima, Tokyo 196–8540, Japan.*
Received October 17, 2007; accepted February 18, 2008; published online February 18, 2008

A method using high-performance liquid chromatography (HPLC) and atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) was established for the detection of 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) and its conjugate with *N*-acetyl-L-cysteine (NAC). The optimal chromatographic conditions were obtained on an ODS column (150×4.6 mm, 3 μm) with the column temperature at 37 °C. The mobile phase consisted of a methanol-0.1% trifluoroacetic acid (TFA) mixture (50 : 50, v/v), and the flow rate was 0.3 ml/min. The detection wavelength was set at 220 nm. The identities of the peaks were accomplished by comparing retention times (t_R), UV and mass data. All calibration curves showed good linear regression (correlation coefficients for 6-MITC and NAC>0.999) within test ranges. The developed method provided satisfactory precision calculated as percent coefficient of variation with overall intra-day and inter-day variations of less than 5% (4.1 and 4.9% for 6-MITC; 4.2 and 4.9% for NAC). Both 6-MITC and NAC had good responses in the positive APCI and formed strong $[M+H]^+$ ions in the full scan spectra at an *m*/*z* of 206 and 164, respectively. The presence of the $[M+H]^+$ ion for the 6-MITC/NAC conjugate was also observed at an *m*/*z* of 369. To our best knowledge, this is the first report that describes the formation of the 6-MITC/NAC conjugate and its detection method by HPLC-MS.

Key words HPLC-MS; 6-(methylsulfinyl)hexyl isothiocyanate; *N*-acetyl-L-cysteine; wasabi

Previous reports have documented that some isothiocyanates (ITCs) prevent the formation of cancers in animal models.[1—4] 6-(Methylsulfinyl)hexyl isothiocyanate (6-MITC) is found in wasabi (*Wasabia japonica*), a Japanese indigenous herb, and a variety of studies on the anticancer properties of 6-MITC have been described,[5,6] with 6-MITC attracting great attention as a new possible candidate for controlling cancer cell progression and metastasis. Anticancer activity of 6-MITC *in vitro* was studied with a human cancer cell (HCC) panel and 6-MITC directly affected the cells in the HCC panel.[5] A suppression was found in the growth and survival of the cells in culture, and the affected cells were very specific, which included breast cancer and melanoma cell lines. A "COMPARE" analysis using a computerized algorithm, which was based on the HCC database, suggested the suppression mechanism of 6-MITC is unique, involving multiple pathways, and may be different from those of other known chemicals. Another 6-MITC-related anticancer study involves the oral administration of 6-MITC and its effects on the macroscopic pulmonary metastasis. Murine B16-BL6 melanoma cells were injected subcutaneously or intravenously in C57BL/6J mice and the effects of 6-MITC on the metastatic foci formation in the lungs were analyzed.[6] The number of metastasized cells in the lungs was significantly reduced by the administration of 6-MITC, and 56% of foci formation was inhibited by the 2 week-prior administration, whereas only 27% inhibition was obtained by the concomitant administration with the cancer cell injection.

Anti-cancer activities of ITCs largely involve modulation of metabolism for carcinogens through inhibition of the phase 1 enzymes and/or induction of the phase 2 enzymes, such as glutathione S-transferase and quinone reductase (QR).[7—12] In our previous study, we reported that 6-MITC induced QR activity in Hepa1c1c7 cells in a dose-dependent manner.[13] However, when the dose level was high at 80 μM, the effect of 6-MITC was nullified and it significantly reduced QR activity. Augmentation of QR activity by 6-MITC seemed to be due to the increase in its mRNA expression, and the inhibition of its activity at 80 μM was ascribed to the reduced gene expression. This negative effect of 6-MITC at 80 μM was overcome by *N*-acetyl-L-cysteine (NAC) treatment but its mechanism is still unknown.

High-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) has been documented as a useful tool for detecting bioactive components, such as flavonoids, puerarin and saponins in herbal medicines,[14—17] and HPLC with an ultraviolet-ray (UV) detector and MS has been applied for determining some of these components.[18,19] Although HPLC-MS is a more expensive and complex option than other HPLC or TLC detectors, it can greatly simplify sample pre-treatment procedures and shorten separation times in HPLC due to the high selectivity and sensitivity of MS detection.[20] As mentioned earlier, the negative effect of 6-MITC on the cells at a higher dosage (80 μM) was overcome by the NAC treatment with an unknown mechanism.[13] In the process of elucidating this mechanism, we hypothesized that 6-MITC and NAC were acting in the cells not independently but in synergy, creating a 6-MITC/NAC conjugate. To prove this point, in the present study, we developed a method using HPLC with a UV detector and MS for detect-

* To whom correspondence should be addressed. e-mail: yamaguch@aomori-u.ac.jp

ing 6-MITC and its conjugate with NAC.

Experimental

Materials and Reagents 6-MITC was obtained from Shiratori Pharmaceutical Co. Ltd. (Chiba, Japan). Analytical grade NAC was purchased from Sigma-Aldrich Japan K. K. (Tokyo, Japan). HPLC grade methanol and trifluoroacetic acid (TFA) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Water was purified using a Milli-Q A10 (Elix5/Synthesis) system (Millipore Co., Tokyo, Japan).

HPLC System and Conditions The HPLC analysis was performed on a Hewlett-Packard 1100 system (Tokyo, Japan) equipped with a degasser, dual pump, autosampler and UV detector. An Inertsil® ODS-3 column (150×4.6 mm, 3 μm; GL Sciences Inc., Tokyo, Japan) at 37 °C was used for all analyses. The mobile phase consisted of a methanol-0.1% TFA mixture (50 : 50, v/v), and the flow rate was 0.3 ml/min. The injection volume was 10 μl, and all sample solutions were filtered through a 0.45 μm membrane filter prior to the HPLC analysis.

HPLC-MS System and Conditions The MS analysis was performed on an JMS-LCmate (MS-BU30) LSMS system (JEOL Ltd., Tokyo, Japan) equipped with an atmospheric pressure chemical ionization (APCI) interface. The HPLC-MS/APCI analysis was conducted with a positive and full scan mode, and the mass range was set at 20—1500 Da. The conditions of the APCI source were as follows: nebulizing gas (N_2) flow rate, 1.5 l/min; interface temperature, 500 °C; needle voltage, 5 kV; orifice1 temperature, 100 °C; orifice1 voltage, 10 V; ring voltage, 43 V; ion guide voltage, 2.5 kV. Data were acquired and analyzed with LC/MS MS-MP30 software version 1.8.00 (JEOL Ltd., Tokyo, Japan).

Calibration Curves, Limits of Detection and Quantification Stock solutions for 6-MITC and NAC were prepared and diluted to appropriate concentrations for the construction of calibration curves. Six concentrations (concentration ranges of 103—5125 μg/ml for 6-MITC and 81—4071 μg/ml for NAC) of the standard solution were injected in triplicate. The calibration curves by a least-squares linear regression were constructed by plotting the mean peak areas *versus* the concentration of standards. The lowest concentration of working solution was diluted with methanol to yield a series of appropriate concentrations, and the limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions were separately determined as a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Intra- and Inter-day Precision and Stability Intra- and inter-day variations were analyzed to determine the precision of this newly developed method. The intra-day variation was determined by analyzing the same standard solution for 6-MITC or NAC in triplicate for three different times on the same day. For inter-day variation, the solution was examined in triplicate for three consecutive days. Stability of the 6-MITC standard solution was tested at 4 °C every 8 h over 48 h. The long-term stability was also assessed after the standard solution was stored at −20 °C for 30 d. The freeze-thaw stability was determined after three freeze-thaw cycles (from −20 to 20 °C) on three consecutive days. Room temperature stability was assessed keeping the standard solution at room temperature (about 25 °C) under room light for 12 h. All the stability analyses were performed in triplicate. The relative standard deviation (RSD) was taken as a measure of precision and stability.

Detection of the 6-MITC/NAC Conjugate A mixture of 6-MITC (25 mM) and NAC (25 or 625 mM) was prepared and an aliquot (10 μl) was immediately analyzed by HPLC (0 h time point), determining the peak area. The remainder of the mixture was incubated at 37 °C for 0.5, 1 and 3 h; a 10 μl aliquot was analyzed by HPLC. When a peak, other than that of 6-MITC or NAC, was observed by HPLC in the incubated mixture, a peak examination was performed by MS and its *m*/*z* was analyzed.

Results and Discussion

HPLC For monitoring of thiols and ITCs, an isocratic mobile phase consisting of 0.1% TFA in water : acetonitrile (60 : 40, v/v) has been reported to produce decent results.[21] Several tests, including gradient and isocratic separations, were performed for optimizing the flow rate and compositions of the mobile phase to achieve a high-quality chromatographic peak shape and resolution. As a result, 0.05% TFA in 50% methanol with a flow rate of 0.3 ml/min was chosen as the eluting solvent system to give the desired separation and acceptable tailing factor within the running time of 40 min.

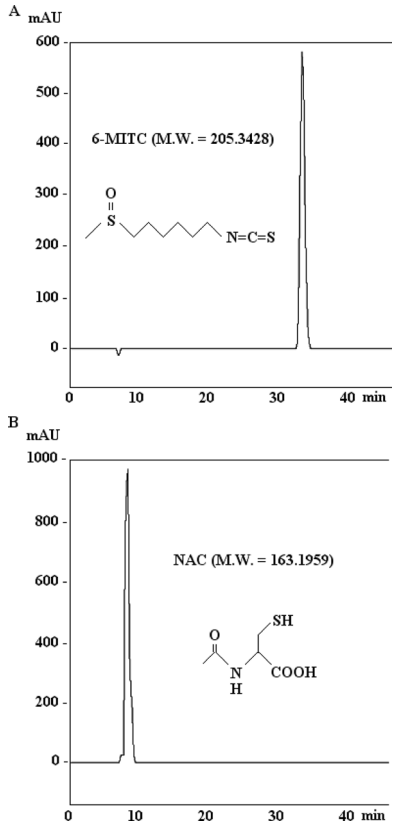

Fig. 1. Typical HPLC Chromatograms and Structures
(A) 6-MITC; (B) NAC.

The injection needle was washed with methanol before each injection to prevent sample-to-sample contamination, and no contamination was detected in each chromatogram. Although 6-MITC and NAC show slightly different UV absorption properties, it was found that the compounds could be monitored in a single run at 220 nm after various detection wavelengths were examined. Representative chromatograms and structures for 6-MITC and NAC are shown in Figs. 1A and B. The retention times (t_R) were about 33 min for 6-MITC and 9 min for NAC.

HPLC-MS/APCI Since chromatographic peaks could not be identified unambiguously by only t_R and UV spectra in a considerable complex matrix, HPLC-MS was used for the confirmation of peak identification. Under fixed HPLC conditions, the kind of ionization interface is a major control for maximizing the detector response efficiency and improving the peak appearance.[20] APCI is widely used as an atmospheric pressure ionization technique, especially for neutral compounds, in which ion suppression by contaminants less frequently occurs compared to electrospray ionization. In APCI, the corona discharge can provide a source of electrons in the gas-phase. This can be advantageous for some compounds that can undergo electron capture. In positive MS/APCI, the introduction of proton-affinitive atoms, such as oxygen and nitrogen, to the analyte is very effective in increasing the sensitivity of the resulting derivative.[22] In the present study, 6-MITC and NAC were analyzed by direct-flow injection to optimize the MS conditions, and thereafter, they were analyzed by HPLC-MS. First, HPLC-MS/ESI was conducted but the results were not preferable. Therefore,

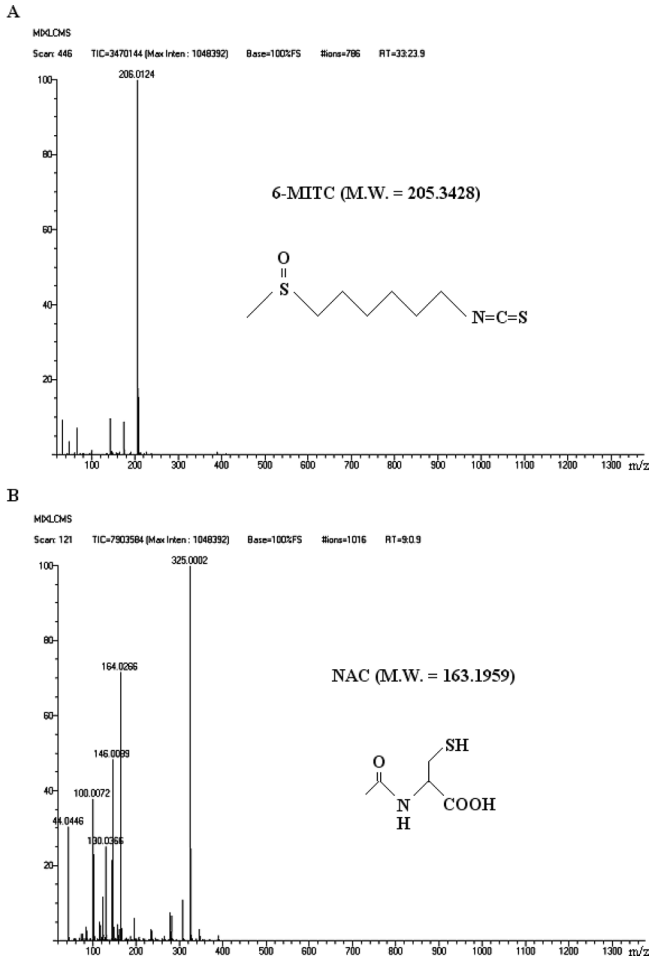

Fig. 2. Product Ion Mass Spectra and Structures
(A) 6-MITC; (B) NAC.

HPLC-MS/APCI was conducted as an alternative method. Both 6-MITC and NAC had good responses in the positive APCI and formed strong $[M+H]^+$ ions in the full scan spectra at an *m*/*z* of 206 and 164, respectively (Figs. 2A, B). The ion at an *m*/*z* of 325 observed in Fig. 2B is perhaps formed by the presence of *N*,*N*′-diacetyl-L-cysteine (MW=324.38) derived from NAC.

Calibration Curve, Sensitivity and Precision Calibration was performed by a least-squares linear regression of the peak areas *versus* the standard 6-MITC or NAC concentrations. Visual inspection of the plotted calibration curves and correlation coefficients (0.9997 for 6-MITC and 0.9993 for NAC) confirmed that the calibration curves were linear over the examined concentration ranges of 103—5125 μg/ml for 6-MITC and 81—4071 μg/ml for NAC. LOD was 0.2 μg/ml (1 μM) for 6-MITC and 0.16 μg/ml (1 μM) for NAC. LOQ was 1.03 μg/ml (5 μM) for 6-MITC and 0.81 μg/ml (5 μM) for NAC. These results indicate that 6-MITC can be detected and quantified at 1 μM and 5 μM, respectively. As we have previously reported, the negative effect of 6-MITC on cells was found at 80 μM,[13] and Ye *et al.* have reported that ITCs in plasma and serum can be detected at 1 μM.[23] These earlier reports and our present results suggest that the methods in the present study can be applied to analyzing effects of 6-MITC both *in vitro* and *in vivo*. Furthermore, the intra- and inter-day precisions calculated as percent coefficient of variation were less than 5% (4.1 and 4.9% for 6-MITC; 4.2 and 4.9% for NAC) for both 6-MITC and NAC. These results indicate that the methods in the present study are valid for an accurate and sensitive detection of 6-MITC or NAC.

Stability of 6-MITC The stability tests in the present study were designed for the anticipated conditions that may be experienced in the clinical environment. The stability of the 6-MITC standard solution was tested at 4 °C every 8 h over 48 h, and the long-term stability was also assessed after the standard solution was stored at −20 °C for 30 d. Furthermore, the freeze-thaw stability was determined after three freeze-thaw cycles (from −20 °C to 20 °C) on three consecutive days. Room temperature stability was also assessed keeping the standard solution at room temperature (about 25 °C) under room light for 12 h. RSDs for all the stability analyses were less than 4%. These results revealed that the stability of 6-MITC was within the acceptable range. In fact, in light of 6-MITC attracting great attention as a new possible candidate for controlling cancer cell progression and metastasis, the results of these stability tests are very appealing for practical use.

Detection of the 6-MITC/NAC Conjugate A mixture of 6-MITC (25 mM) and NAC (625 mM) was prepared and an aliquot (10 μl) was immediately analyzed by HPLC (0 h time point), determining the peak area. The remainder of the mixture was incubated at 37 °C for 0.5, 1 and 3 h; a 10 μl aliquot was analyzed by HPLC. Figure 3 shows a typical series of chromatograms for the decrease in free 6-MITC (t_R of 33 min, $Y=-2.522X+24.998$, where Y represents the estimated concentration (mM) of 6-MITC and X represents the incubation time (h), Estimated half life of 6-MITC is 4.956 h), which demonstrates the time-dependent formation of the 6-MITC/NAC conjugate (t_R of 13.5 min) as a product. The conjugate gave about half the signal of 6-MITC at 0.5 h, while it was twice as much at 3 h. The t_R for the conjugate appeared shorter than that for 6-MITC due to the hydrophilic group derived from NAC. Although the concomitant decrease in 6-MITC was obvious after 3 h incubation, it is not clear in NAC (t_R of 9 min) (Fig. 3, lower left), and two peaks for NAC were observed, perhaps because the initial concentration of NAC was very high (625 mM). A lower initial concentration (25 mM) of NAC was also examined for the detection of the 6-MITC/NAC conjugate, however, the reaction between 6-MITC and NAC was slow and the concomitant formation of the 6-MITC/NAC conjugate was very little even after 3 h incubation (Fig. 3, lower right). The mixture of 6-MITC and NAC was also analyzed by MS for confirmation of peak identification. Figure 4A shows product ion mass spectra for the 6-MITC and NAC mixture at a t_R of 13.5 min. A possible structure of the conjugate is also shown. The $[M+H]^+$ ions for 6-MITC and NAC were observed at an *m*/*z* of 206 and 164, respectively, and the ion at an *m*/*z* of 369 represented the $[M+H]^+$ ion for the 6-MITC/NAC conjugate. The ion at an *m*/*z* of 305 was probably formed by cleavage at the broken line (Fig. 4A), or it could be a conjugate formed by 6-MITC and a fragment of NAC. Figure 4B is typical mass chromatograms of the mixture and the $[M+H]^+$ ion (*m*/*z* of 369) for the 6-MITC/NAC conjugate exhibiting the highest peak at a t_R of 13.5 min. These data indicate that the 6-MITC/NAC conjugate (*m*/*z* of 369, t_R of 13.5 min) is formed as a product in the mixture of 6-MITC and NAC.

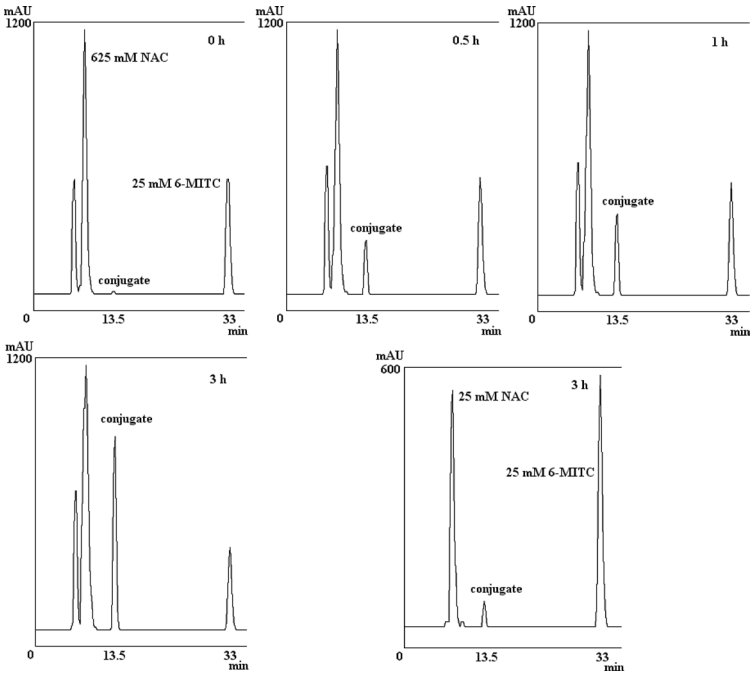

Fig. 3. Time Course Formation of 6-MITC/NAC Conjugate

A typical series of chromatograms for the decrease in free 6-MITC (t_R of 33 min), which demonstrates the time-dependent formation of the 6-MITC/NAC conjugate (t_R of 13.5 min) as a product. Although the concomitant decrease in 6-MITC is obvious after 3 h incubation, it is not clear in NAC (t_R of 9 min) (lower left), and two peaks for NAC were observed, perhaps because the initial concentration of NAC was very high (625 mM). A lower initial concentration (25 mM) of NAC is also examined for the detection of the 6-MITC/NAC conjugate, however, the reaction between 6-MITC and NAC is slow and the concomitant formation of the 6-MITC/NAC conjugate is very little even after 3 h incubation (lower right).

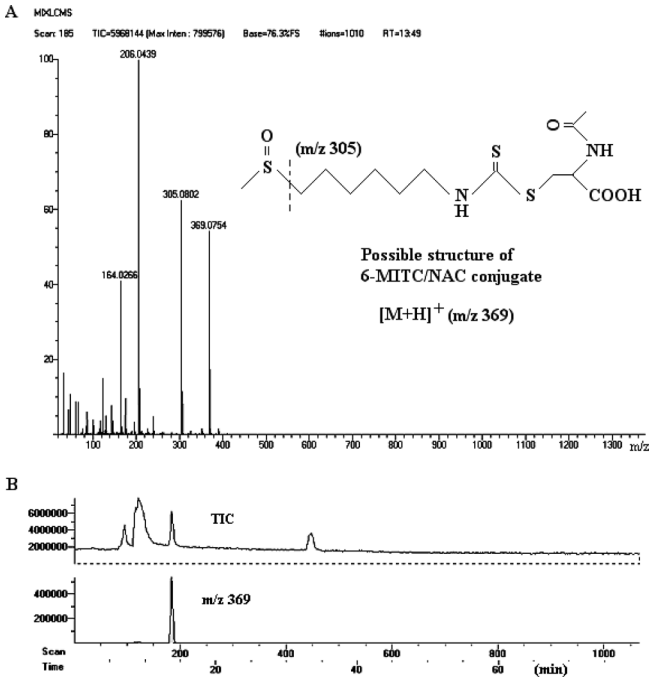

Fig. 4. Detection of 6-MITC/NAC Conjugate

(A) Product ion mass spectra and possible structure; (B) Ion chromatograms of total ions and ion with an *m/z* of 369. The conditions of the APCI source are as follows: nebulizing gas (N_2) flow rate, 1.5 l/min; interface temperature, 500 °C; needle voltage, 5 kV; orifice1 temperature, 100 °C; orifice1 voltage, 10 V; ring voltage, 43 V; ion guide voltage, 2.5 kV. Data are acquired and analyzed with LC/MS MS-MP30 software version 1.8.00 (JEOL Ltd., Tokyo, Japan).

Conclusions

In the present study, an HPLC-MS/APCI method for the detection of 6-MITC and its conjugate with NAC has been developed. This method is validated for good accuracy, repeatability and precision. To our best knowledge, this is the first report that describes the formation of the 6-MITC/NAC conjugate. We have previously reported that the negative effect of 6-MITC on cells at a higher dosage (80 μM) was overcome by NAC treatment with an unknown mechanism.[13] In the process of elucidating this mechanism, we hypothesized that 6-MITC and NAC were acting in the cells not independently but in synergy, creating a 6-MITC/NAC conjugate. Our present study proceeded with this hypothesis, which is a rational strategy to elucidate the synergic effects of 6-MITC and NAC, and the newly developed method will be a powerful tool for understanding the mechanism underlying the biological effects of 6-MITC.

Acknowledgements This study was partly supported by a Grant-in-Aid from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

1) Lin J. M., Amin A., Trushin N., Hecht S. S., *Cancer Lett.*, **74**, 151—159 (1993).
2) Zhang Y., Kensler T. W., Cho C. G., Posner G. H., Talalay P., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3147—3150 (1994).
3) Fuke Y., Haga Y., Ono H., Nomura T., Ryoyama K., *Cytotechnology*, **25**, 197—203 (1997).
4) Yano T., Yajima S., Virgona N., Yano Y., Otani S., Kumagai H., Sakurai H., Kishimoto M., Ichikawa T., *Cancer Lett.*, **155**, 115—120 (2000).
5) Nomura T., Shinoda S., Yamori T., Sawaki S., Nagata I., Ryoyama K., Fuke Y., *Cancer Detect. Prev.*, **29**, 155—160 (2005).
6) Fuke Y., Shinoda S., Nagata I., Sawaki S., Murata M., Ryoyama K., Koizumi K., Saiki I., Nomura T., *Cancer Detect. Prev.*, **30**, 174—179 (2006).
7) Prochaska H. J., Santamaria A. B., Talalay P., *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2394—2398 (1992).
8) Maheo K., Morel F., Langouet S., Kramer H., Le Ferrec E., Ketterer

B., Guillouzo A., *Cancer Res.*, **57**, 3649—3652 (1997).
9) Singletary K., MacDonald C., *Cancer Lett.*, **155**, 47—54 (2000).
10) Hou D. X., Fukuda M., Fujii M., Fuke Y., *Cancer Lett.*, **161**, 195—200 (2000).
11) Hou D. X., Fukuda M., Fujii M., Fuke Y., *Int. J. Mol. Med.*, **6**, 441—444 (2000).
12) Nakamura Y., Ohigashi H., Masuda S., Murakami A., Morimitsu Y., Kawamoto Y., Osawa T., Imagawa M., Uchida K., *Cancer Res.*, **60**, 219—225 (2000).
13) Ryoyama K., Mori N., Nara M., Kidachi Y., Yamaguchi H., Umetsu H., Fuke Y., *Anticancer Res.*, **23**, 3741—3748 (2003).
14) Wang X. M., Sakuma T., Adjaye E. A., Shui G. K., *Anal. Chem.*, **71**, 1579—1584 (1999).
15) Li W. K., Gu C. G., Zhang H. J., Wang D. V. C., Fitzloff J. F., Fong H. H. S., Breemen R. B. V., *Anal. Chem.*, **72**, 5417—5422 (2000).
16) Yan S. K., Xin W. F., Luo G. A., Wang Y. M., Cheng Y. Y., *Chem. Pharm. Bull.*, **53**, 1392—1395 (2005).
17) Hu P., Luo G. A., Zhao Z. Z., Jiang Z. H., *Chem. Pharm. Bull.*, **53**, 705—709 (2005).
18) Yang N. L., Wu Y. J., Cheng Y. Y., *Chin. J. Anal. Chem.*, **31**, 731—734 (2003).
19) He J. T., Shi Z. H., Zhao M. P., Chang W. B., *Chin. J. Anal. Chem.*, **32**, 719—721 (2004).
20) Yu Z. G., Gao X. X., Zhao Y. L., Chen X. H., Bi K. S., *Chem. Pharm. Bull.*, **54**, 588—590 (2006).
21) Conaway C. C., Krzeminski J., Amin S., Chung F. L., *Chem. Res. Toxicol.*, **14**, 1170—1176 (2001).
22) Higashi T., Takido N., Yamaguchi A., Shimada K., *Anal. Sci.*, **18**, 1301—1307 (2002).
23) Ye L., Dinkova-Kostova A. T., Wade K. L., Zhang Y., Shapiro T. A., Talalay P., *Clin. Chim. Acta*, **316**, 43—53 (2002).