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Characterization of Allicin Transformation Products and Determination of Allicin

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Abstract

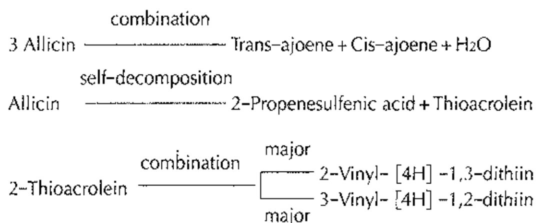
Allicin was synthesized for the purpose of identification and quantitation of a pharmaceutical dosage form in soft capsules. The identified transformed products from allicin were diallyl disulfide, 3-vinyl-[4H]-1,2-dithiin and 2-vinyl-[4H]-1,3-dithiin in gas chromatrographic conditions and diallyl disulfide and ajoene in HPLC. Allicin is thermally unstable, it may be completely decomposed to vinyl dithiin isomers in GC conditions. For that reason, allicin was not found directly in the pharmaceutical dosage forms. In HPLC conditions, mobile phase was methanol/water containing 0.1% formic acid (65/35) and column was μ-Bondapak C_{18}. Detection wavelength was 254nm. The retention time of allicin was 6.98 min. The calibration range for allicin was 10 μg/ml to 200μg/ml and correlation coefficient (r) was 0.987.

Key words : allicin transformation, garlic, alliin, determination of allicin

INTRODUCTION

Garlic has been used as a folk medicine for the treatment of various diseases, infections, vascular disorders and for the prevention of heart diseases and rheumatism[1,2]. The antibacterial effect of garlic was scientifically reported by Louse Pasteuer and confirmed by Cavallito *et al.*[3-5]. Stoll and Seeback[6] reported that intact garlic cloves contains 0.24% by weight S-allylcystenis-S-oxide called alliin, a colorless and odorless solid crystal, which converts into allicin, pyruvic acid and ammonia by an enzyme called allinase. Block *et al.*[7,8] reported that sulfur compounds extracted from garlic depend on the temperature and the solvent. Small *et al.*[7] and Block *et al.*[8] have also suggested that decomposition of allicin proceeds on several pathways as shown in Scheme 1.

Recently, many of the garlic products have been marketed as health food with purported nutrient and tonic effects to human beings. The quality of garlic products is hard to control. Therfore, the development of an assay method for controlling the quality and evaluation of the safety of commercial garlic products is required. Since, allicin is a main components of garlic flavor and a marker constituent of garlic products, the determination of allicin is expected to be essential for the quality control of garlic products. For the analysis of allicin and/or its related compounds, some methods-for example, a semi-quantitative paper chromatographic technique, a method[9] for the assay of thiosulfinate using the color reaction for pyruvic acid and oxygen flask combustion method-were employed.



Scheme 1. Decomposition pathways of allicin.

The increasing biological interests in garlic and its different constituents in commercial products brought

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up the need for a rapid and reproducible method to evaluate and standardize the allicin content in the commercial products. Gas chromatography (GC) and Gas chromatography/Mass Spectrometry (GC/MS) are preferable method on the basis of specificity and sensitivity. Allicin is a volatile organic compound containing sulfur and is not stable even at room temperature. During separation process in the GC, allicin undergoes dehydration and forms two isomeric disulfides[10]. Since the formation ratio of these isomeric vinyl dithiin is proportional to the allicin content in the sample, allicin can be determined indirectly using the isomeric vinyl dithiin as indicators[11,12]. Due to the thermal instability of allicin, it is impossible to determine allicin directly using GC and GC/MS. The High performance Liquid Chromatography (HPLC) made it possible to carry out the determination of allicin in garlic directly as well as allicin containing products[13-16].

The present study describes the identification of allicin related decomposed compounds and the development of an assay method for the determination of allicin in commercial products.

MATERIALS AND METHODS

Reagents and chemicals

Commercially available chemicals were used without further purification. Methanol, acetonitrile, hexane, and tetrahydrofuran for the HPLC'S (J.T) were used and the water, filtered through a Milli-Q system, was used (Millipore Corp.). Four of allicin containing pharmaceutical product which is available as a dosage form (soft capsule) were purchased from a local pharmacy. Diallyl disulfide was obtained from Aldrich. Sep-Pak C_{18} catridges were obtained from Waters Associates. Other chemicals used were of the highest purity grade available and obtained from various suppliers including Aldrich, Simgma and Wako (Osaka, Japan).

Instrumentation

The HPLC system for the UV spectra used for the analysis of allicin related compounds was a HP Model 1090A HPLC equipped with diode-array detector (Hewlett Packard). The analytical column used was Hypersil-ODS (100 × 4.6mm i.d., 5μm partical size, Palo Alto).

The HPLC system for the analysis of allicin was composed of a Young In Model 910 solvent delivery system equipped with a fixed type Young In Model 710 UV detector, a Rheodyne Model 7125 injector (Cortati) equipped with a 20μl sample loop and a Waters Model 745 Data Module (Milford) as an integrator. μ-Bondapak C_{18} (300 × 3.9mm i.d., 10μm particle size, Waters Associates) was used for the separation and the detection wavelength of 254nm was used for quantitative determinations. GC/MS analysis of allicin was performed with an HP 5980 Mass Spectrometer, a quadrupole mass spectrometer, coupled directly to an HP5890A GC. The column was HP fused silica capillary coated with cross-linked 5% phenylmethylsilicone (SE-54, 12m × 0.2mm i.d., 0.33μm film thickness). Oven temperature was programmed from 40 to 150°C at the increasing rate of 10°C/min. and 150°C to 300 °C at the increasing rate of 25°C/min. and stay 10min. at 300°C. Helium was used as carrier gas at a flow rate of 1ml/min. for column flow and 7 ml/min. for septum purge and the split ratio of 1/10. Injector temperature was 280°C and transfer line temperature was 280°C. The mass spectrometer was operated at an ionization potential of 70eV with electron impact mode for the full scan.

Synthesis of allicin

Allicin was synthesized using the procedure of Iberl *et al.*[14,15] and were identified by IR, NMR and MS with direct insertion probe (DIP) mode.

Hydrogen peroxide (30% 2.4ml) in 17.6ml acetic acid was added dropwisely for 30min. to the solution of 0.02mol dially disulfide in 17ml acetic acid at 0°C while stirring. The reaction mixture was allowed to react for 30min. at ambient temperature with vigorous stirring. Further treatment was performed at 4°C. The reaction mixture was neutralized with 10ml of concentrated potassium hydroxide solution and added 10ml of methanol and then extracted three times with 20ml of hexane. The hexane layer was discarded. The polar aqueous portion was made up to 50ml with water and then extracted with 25ml of

ether twice. The ether layer was collected and the aqueous layer was discarded. The combined ether layer was washed with 30ml of 5% $NaHCO_3$ solution three times and then washed again with 30ml of water once. The washed ether layer was filtered through anhydrous sodium sulfate and evaporated using a rotary evaporator. The residue was concentrated under vacuum of around 0.05mmHg for 1 hour to give 1g (31% yield) of crude allicin which was purified by preparative HPLC using hexane/tertrahydrofuran= 90 : 10 with sillica gel column : IR(neat) 1090(S, S =0) ; 1H NMR($CDCl_3$) δ 6.30–5.0(m, 6H), δ 3.9–3.6(m, 4H) ; MS(DIP) molecular weight 162m/z, mass spectra were shown in Fig. 1. The purified allicin was diluted to 0.1% solution with ether and stored at −20° C. The diluted allicin was used as a standard solution.

Preparation of standard solutions

Stock soultions (100μg/ml) of allicin were prepared by diluting the standard allicin with ether. Working solutions were prepared immediately prior to use by serial dilution of the stock solutions with mobile phase.

Preparation of dosage form samples

An allicin sample was prepared by weight. Five soft capsules were weighed first and then transferred to a 50ml beaker. The five empty capsules were weighed and the average weight per capsule was calculated. Allicin of a solution which was an the

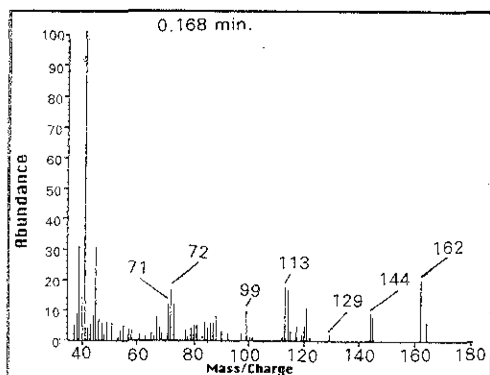

Fig. 1. Electron-impact mass spectra of synthesized allicin using a direct insertion probe-mass spectrometry (DIP-MS).

equivalent to 300μg of was transferred to a 20ml test tube with stopper. After the addition of 3ml water to the test tube, 5ml of hexane was added twice to eliminate the lipid soluble compounds. 1ml of aqueous portion was then applied to Sep–Pak C_{18} cartridge pre–activated with 5ml of methanol twice and 5ml of distilled water twice. After washing with 5ml of distilled water, the sample was eluted with 5ml of methanol. Methanol was evaporated to the volume of around 0.5ml and then make up to the volume of 1ml with methanol, which is filtered with 0.45μm teflon filter and loaded into HPLC system.

Identification of allicin transformation compounds

The identification of allicin and its decomposed compounds was performed using HPLC–diode array detector and GC/MS.

RESULTS AND DISCUSSION

Extraction and recovery of allicin in pharmaceutical dosage forms

Allicin containing pharmaceutical products are composed of various other substances such as lipid soluble vitamins, water soluble vitamins, and vegetable oil. Therefore clean–up procedures before being applied to GC or HPLC are very important to develop an analytical method.

The contents of soft capsules are mixed with water and then washed with hexane to eliminate the lipophilic substances. The aqueous portion was applied to solid phase extraction system using Sep–Pak C_{18} cartridges. The solid phase extraction system has advantages over the liquid extraction steps which results in the formation of emulsions during the analytical steps.

The recoveries for the solid phase extraction system were 97.6% and 91.2% when the applied standard

Table 1. Recoveries of allicin with solid phase extraction step

Concentration, mg/ml	Recoveries*, %	CV, %
0.1	97.6	3.5
0.05	91.2	8.4

*Average of 5 determinations

solution was 0.1mg/ml and 0.05mg/ml and coefficients of variation (CV, %) were satisfied with less than 10% as shown in Table 1.

Characterization of allicin and its transformation products

The GC method was first studied in order to identify allicin with a flame ionization detector (FID). The chromatogram of allicin standard solution is shown in Fig. 2. Unfortunately, four unidentified peaks (three of them are small) were present in the chromatogram. In order to characterize the four unknown peaks in Fig. 2, GC/MS method was employed. The condition of GC/MS was described in a previous earlier section.

The total ion current (TIC) chromatogram is shown in Fig. 3 and its mass spectra of relating TIC are shown in Fig. 4. The result was well matched with the work of Brodnitz *et al.*[10] who indicated the presence of two isomeric cyclic compounds being formed by dehydration of allicin. However, it was hard to find allicin peak. Therefore, the only way to determine the allicin in the GC system is by using the two transformed cyclic isomer. Alternative method was HPLC which is operated at room temperature. For the purpose of identification, a Hewlett-Packard Model 1090 HPLC system with diode array detector was used. Mobile phase was composed of methanol : water containing 0.1% formic acid=65 : 35 with the flow rate of 1.0ml/min. The chromatogram is shown in Fig. 5 and Fig. 6. By the comparison of UV spectra of each peak with the work of Iberl *et al.*[15], four peaks were identified. According to references, there are two isomers of ajoene : (Z)- and (E)-ajoene and ajoene were also transformed from allicin like vinyl dithiins depending on the temperature and the polarity of the solvent.[1,9,14,17,18]

The comparison between GC and HPLC methods showed big differences in the aspect of chromatograms of the methods of interest. There are no allicin and ajoene peaks in GC and no vinyl dithiin peaks in HPLC. This is due to the effect of temperature during the transformation of allicin.

Determination of allicin contents in dosage form

The HPLC method was chosen for the analysis of allicin in the four pharmaceutical dosage forms (soft capsules). Determination of allicin was carried out by the modified method of Iberl *et al.*[15] using a You-

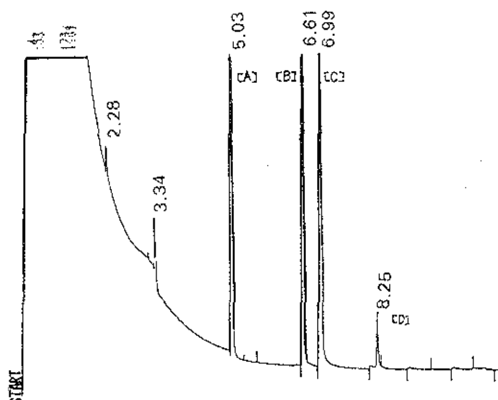

Fig. 2. Capillary gas chromatogram of transformed compounds produced from allicin standard solution.
Idenitification of each peak was confirmed by the results of GC/MS. peak [A] : diallyl disulfide, peak [B] : 3-vinyl-[4H]-1,2-dithiin, peak [C] : 2-vinyl-[4H]-1,3-dithiin, and peak [D] : unknown.

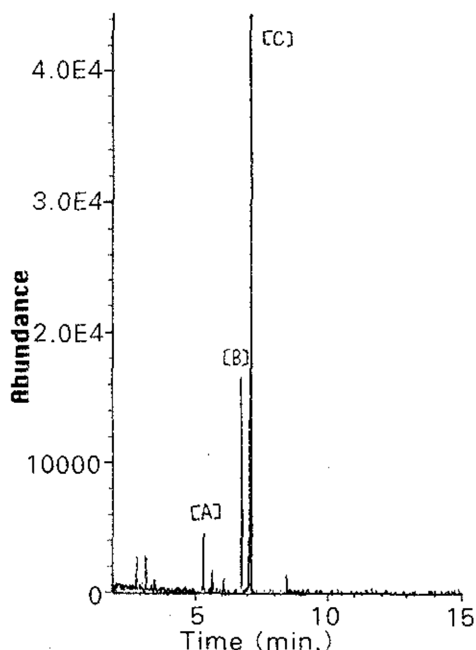

Fig. 3. Total ion current (TIC) chromatogram of electron-impact mass spectrometry (EI-MS) of volatile compounds produced from allicin standard solution.
peak [A] : diallyl disulfide, peak [B] : 3-vinyl-[4H]-1,2-dithiin, and peak [C] : 2-vinyl-[4H]-1,3-dithiin.

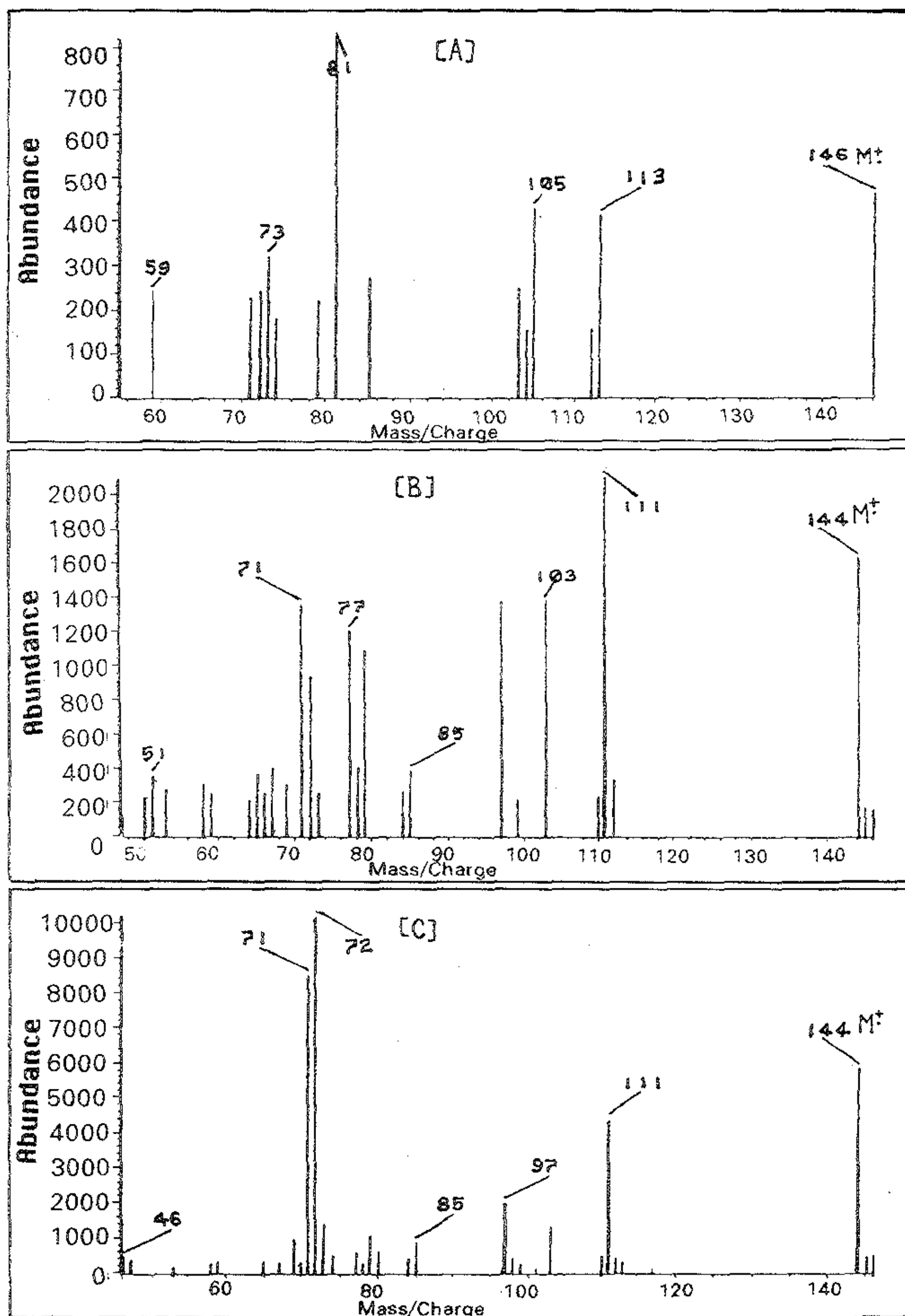

Fig. 4. Mass spectra of each peak.
peak [A] : diallyl disulfide, peak [B] : 3-vinyl-[4H]-1,2-dithiin, and peak [C] : 2-vinyl-[4H]-1,3-dithiin.

ng In Model 910 HPLC sysem. Mobile phase was methanol : water containing 0.1% formic acid=65 : 35 with the flow rate of 1.0ml/min. The retention time of allicin was 6.98 min. as shown in Fig. 7.

The calibration range for allicin was 10μg/ml to 200μg/ml under the established HPLC conditions. Using spiked samples of allicin, accuracy of the method was shown to be in the less than 5% range and precision (% RSD) in the less than 8% range across the concentration range studied. Linear regression analysis of peak area versus allicin concentration gave correlation coefficients (r) of 0.987 (n=3). The prescri-

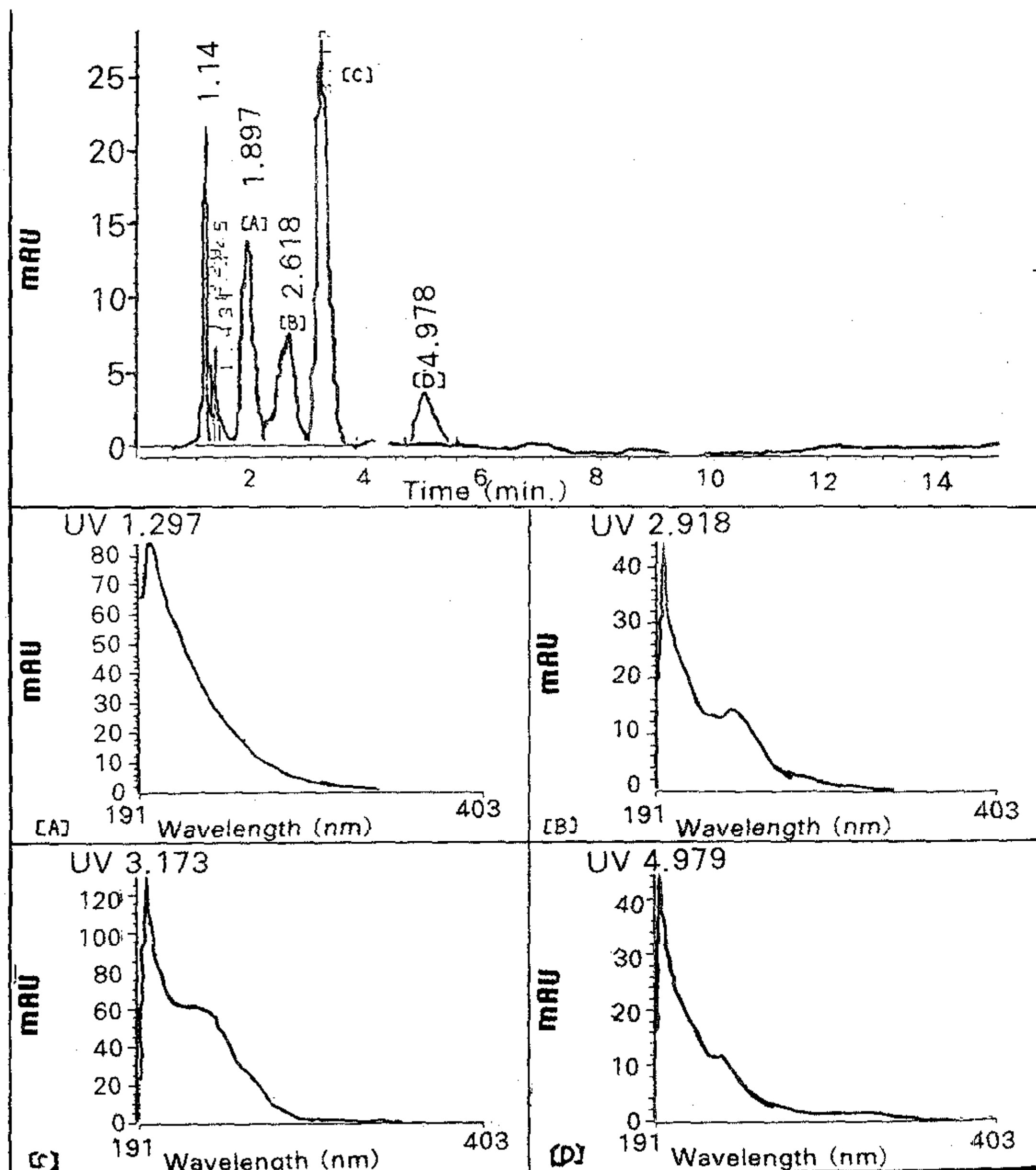

Fig. 5. HPLC chromatogram and UV spectra of transformed compounds produced from allicin standard solution.
peak [A] : diallyl disulfide, peak [B] : ajoene, peak [C] : allicin, and peak [D] : unknown.

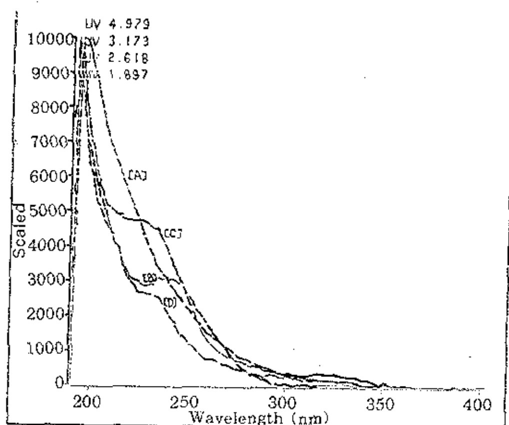

Fig. 6. The overlapping spectra of four peaks relevant to UV spectra of each peak.
peak [A] : diallyl disulfide, peak [B] : ajoene, peak [C] : allicin, and peak [D] : unknown.

ption of each dosage forms are illustrated in Table 2. The chromatogram of a pharmaceutical dosage form sample was shown in Fig. 7 and the analytical results are summarized in Table 3.

Table 2. The prescription of pharmaceutical dosage forms

Product	Contents
A	Garlic oil, α-tocopherol
B	Garlic oil, α-tocopherol, soy lecithin
C	Garlic oil, α-tocopherol, soy lecithin
D	Allicin, α-tocopherol, procain . HCl, retinal palmitate, vitamin B complex, etc.

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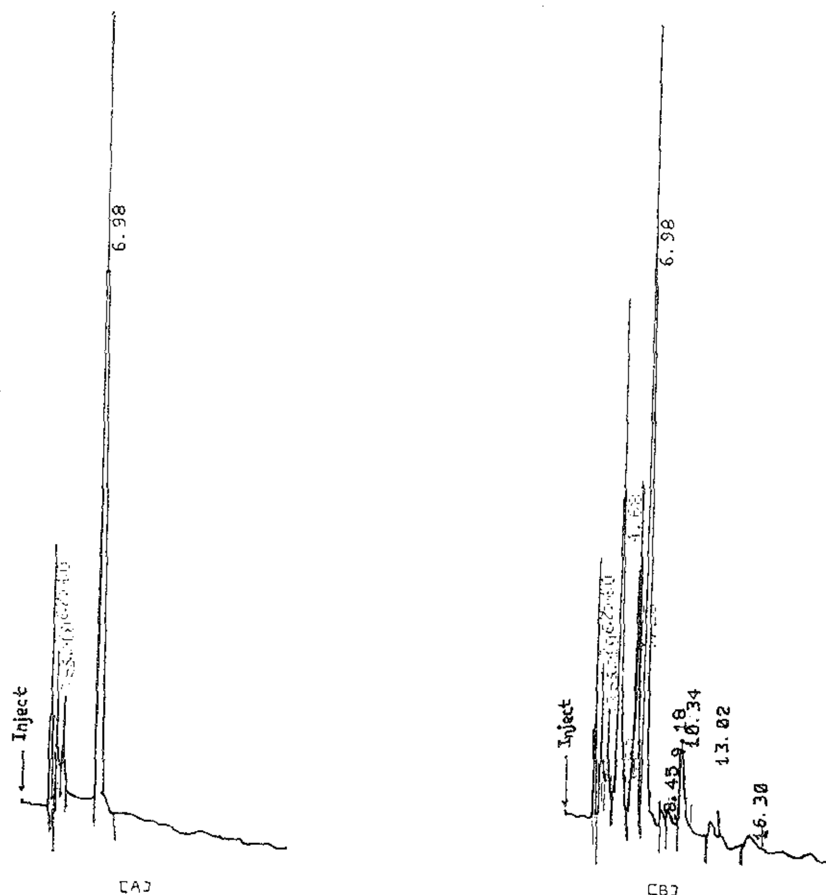

Fig. 7. Typical HPLC chromatograms of allicin standard solution and pharmaceutical dosage forms.
Column was μ-Bondapak C_{18}(300×3.9mm i.d., 10μm) and detection wavelength was 254nm. [A] : allicin standard solution (100ug/ml), [B] : pharmaceutical dosage form sample.

Table 3. Analysis of allicin in pharmaceutical dosage forms

Product	Labeled of amount of allicin (μg)	Amount* found (μg)	% of labeled amount	C.V. (%)
A	300	310.4	103.5	2.80
B	300	287.6	95.92	3.76
C	300	292.1	97.41	4.90
D	100	97.3	97.35	6.68

HPLC conditions : column ; 10μm μ-Bondapak C_{18}(30cm×4.6mm i.d.)
Mobile phase ; methanol/water containing 0.1% formic acid=65 : 35
Flow rate : 1.0ml/min
Detector : UV 254nm
*Average of 3 determinations

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알리신 변형체의 특성과 알리신의 정량

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요 약

마늘 제제중의 주성분인 allicin의 확인 및 정량을 목적으로 standard allicin을 합성하여 direct insertion Prove-Mass Spectrometry (DIP-MS)의 Mass spectra로 확인하였다. Allicin의 transformation products인 diallyl disulfide, 3-vinyl-[4H]-1,2-dithiin과 2-vinyl-[4H]-1,3-dithiin은 cross-linked 5% phenylmethylsilicone capillary column을 사용하여 oven 온도를 40°C에서 150°C까지 10°C/min.으로 상온시킨 후 150°C에서 300°C까지 25°C/min.으로 조절하여 Gas Chromatography-Flame Ionization Detector 및 Gas Chromatography-Mass spectrometry로 확인한 바 그 retention time은 각각 5.03, 6.61, 6.99이었다. 또 다른 transformation product인 ajoene은 Hypersil-ODS column과 65 : 35 비율의 methanol : water containing 0.1% formic acid를 이동상으로 한 High Performance Liquid Chromatography를 이용하여 비교 확인하였다. Allicin은 그 transformation products인 3-vinyl-[4H]-1,2-dithiin과 2-vinyl-[4H]-1,3- dithiin의 비율로 간접 정량하므로, 마늘 제제중 allicin 정량은 solid phase extraction한 후 μ-Bondapak C_{18} column을 이용한 HPLC로 정량한 결과 비교적 양호한 결과를 얻었다.