DETERMINATION OF THYMOL FROM VARIOUS FORMULATIONS BY GAS CHROMATOGRAPHY

M. GRIMS & SENJKUVIC[1] have reported a gas chromatography (G.C.) method to estimate thymol from the extract of thymus vulgaris. Pocaro[2], and Moshonas[3] have described a G.C. method to determine thymol from essential oils. Metthew[4] has described a G.C. method to estimate thymol from blood plasma or serum. No G.C. method has been reported in the literature to estimate thymol in its formulations. In the present work, we have developed the gas chromatographic method to determine thymol from five commercial formulations.

Experimental

Intersmat GC–112 single column gas chromatograph was used, which was equipped with flame ionization detector and 1 mV linear recorder. A 2 meter glass column with 3 mm internal diameter, packed with 3% OV225 on chromosorb (HP) 80/100 mesh was used for all determinations with column oven at 170° C and detector and injection port at 210° C. The carrier gas, nitrogen, flow rate was 32 ml/min. The flow rate of hydrogen was about 20 ml/min and that of air was 400 ml/min.

Standard thymol solution had 50 mg of thymol in 50 ml of chloroform. *Internal standard*: *p*-chlorophenol (about 300 mg) was dissolved in 100 ml of chloroform. (In the case of cream and ointment, *p*-chlorophenol was dissolved in 0·1 N sodium hydroxide solution). *Working standard*: 1 ml of standard thymol solution and 1 ml of internal standard in chlororofrm were diluted to 10 ml with chlororofrm.

Preparation of sample solution

In the case of tablets, thymol was extracted in chloroform after adding the internal standard. In the case of aqueous solutions, thymol was extracted with chloroform after adding 5 ml of 5% sodium bicarbonate and the internal standard in chloroform. In the case of creams and ointments, the sample was mixed with 10% sodium hydroxide and internal standard in sodium hydroxide and heated on a water bath. Thymol was then extracted with chloroform after acidification. The final concentration of thymol was adjusted to about 100 μg/ml with chloroform.

Chromatography

The attenuation was set at 16 and sensitivity at 10^{-11} A/mV. About one microlitre of working standard solution was injected using a 10 microlitre Hamilton Syringe. The thymol peak had a retention time of about 4 min whereas *p*-chlorophenol peak had retention time of about 6 min. Under the same conditions one microlitre of each of the sample solution was injected. The thymol was estimated from the peak heights. Table I gives the results.

TABLE I

Thymol in commercial preparations by the present G.C. method

Product	Amount of thymol present	Found per cent of labelled amount
Tablet	5·4 mg/tab.	100
Aq. solution	0·06%	98
Aq. solution	0·23%	96
Cream	0·50%	96
Ointment	1·0%	99

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SELENIUM—POWERFUL INHIBITOR OF XANTHINE OXIDASE ACTIVITY

SELENIUM, as an oxidant, possesses the unique property of aiding both, hydrogen removal as well as oxygen addition in the oxidative mechanisms of the metabolic processes. As a rapid and most versatile nucleophile. it is considered to amplify and orient SH ⇌ — S — S — interaction[1]. Gout, a metabolic disorder, is characterised by an increase in the xanthine oxidase (xanthine-oxygen oxidoreductase, EC 1.2.3.2) activity, leading to excessive formation of uric acid[2]. The presence and active participation of SH groups at the active site of xanthine oxidase is very well documented[3,4]. Deficiency of serum SH groups is known to characterize many types of arthritis, including gouty arthritis and gout[5]. Moreover, selenium has been used in the treatment of arthritis[6] and has also been reported by Schroeder and Mitchener[7] to result in the lowering of the serum uric acid level in experimental animals. The above considerations led to the study of the interaction of selenium with xanthine oxidase.

Milk xanthine oxidase was prepared by the method of Massey *et al.*[8]. The enzyme activity was measured by the colorimetric method of Owen[9], using Thunberg evacuated tubes, containing in the side arm, 3×10^{-4} M xanthine as substrate. The enzyme (22·5 units*), was preincubated with varying concentrations of sodium selenite for 30 min and then the substrate was mixed to initiate the reaction. The contents were incubated at 37° C and the colour of the formazan formed from 2,3,5-triphenyl tetrazolium chloride was measured at 495 nm. For the determination of time-activity relationship, readings were taken at 30 second intervals. The inhibitor constant and the nature of inhibition were determined by terminating the reaction at 4 minutes.

increasing the concentration of selenium, the inhibition was found to increase exponentially (Fig. 1). The Ki determined was of the order of $2{\cdot}3 \times 10^{-2}\ \mu$M, suggesting a powerful inhibitory activity.

The initial inhibition of xanthine oxidase activity by selenium is attributed to the presence of selenium as toxic selenite (Se^{4+}) ions, which inhibit SH interaction[10] at the active site. The mechanism of reversal of the initial inhibition is due to the apparently ready and reversible conversion of the selenite (Se^{4+}) to selenide (Se^{2-}), the latter being nontoxic[11].

Rapid restoration of the activity of the selenium inhibited enzyme with the addition of glutathione was observed (Fig. 2). This finding indicates the site of selenium inhibition to be the SH groups of the enzyme.

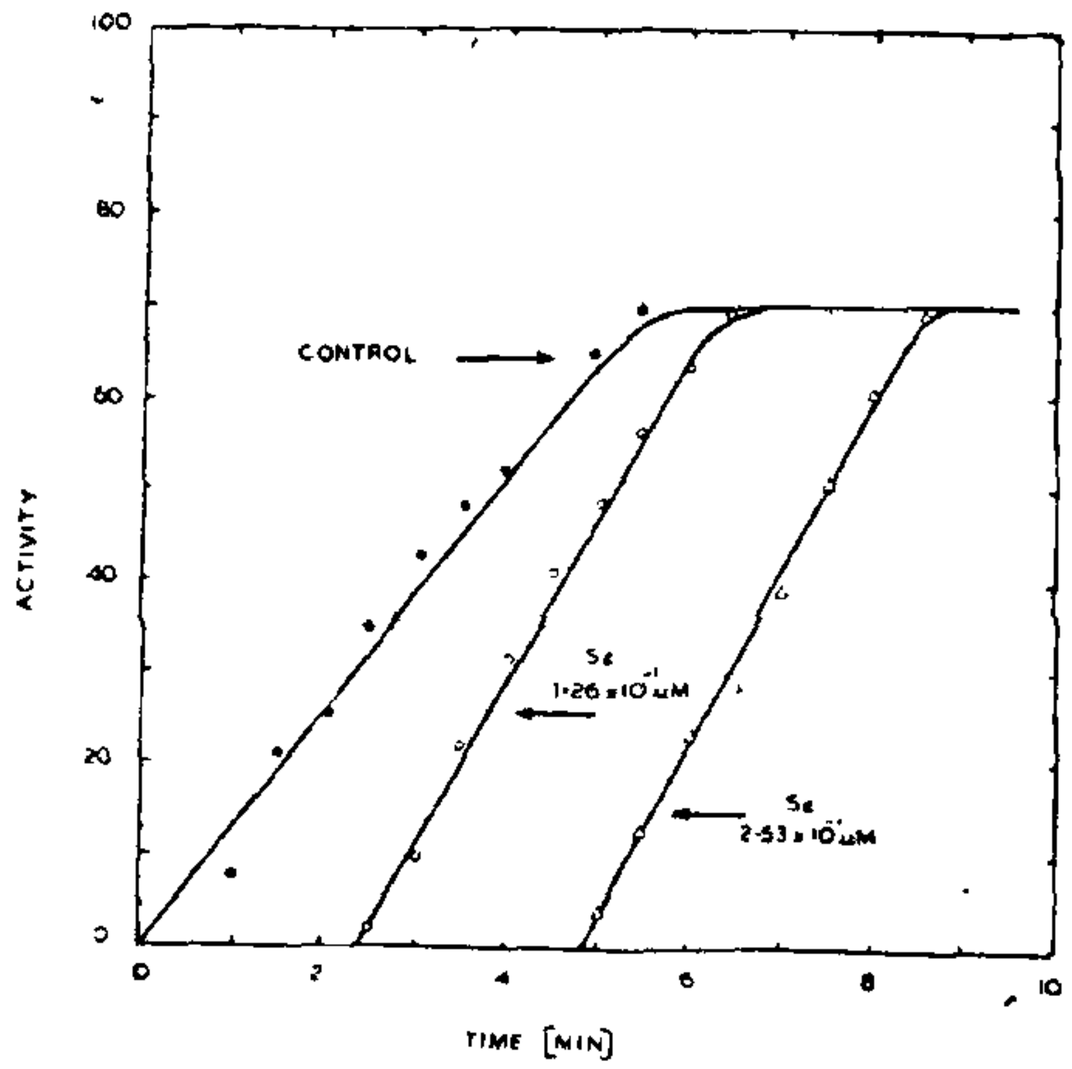

FIG. 1. Effect of seleninum on milk xanthine oxidase activity. Activity is expressed as the amount of formazan formed. —●—, control; —○— $1{\cdot}26 \times 10^{-1}$ μM selenium as sodium selenite; —△— $2{\cdot}53 \times 10^{-1}$ μM selenium as sodium selenite. The reaction mixture in the tube of Thunberg apparatus contained phosphate buffer (0·5 M, PH 7·4), 1·0 ml; 2, 3, 5-triphenyl tetrazolium chloride (8·5 mg), 0·5 ml; xanthine oxidase (22·5 units), 0·1 ml and selenium in the concentration mentioned. Into the lid was placed xanthine (3×10^{-4} M), 0·6 ml and the final volume was adjusted to 5·0 ml. The samples were incubated at 37° C and the reaction was terminated by the addition of 5 ml glacial acetic acid.

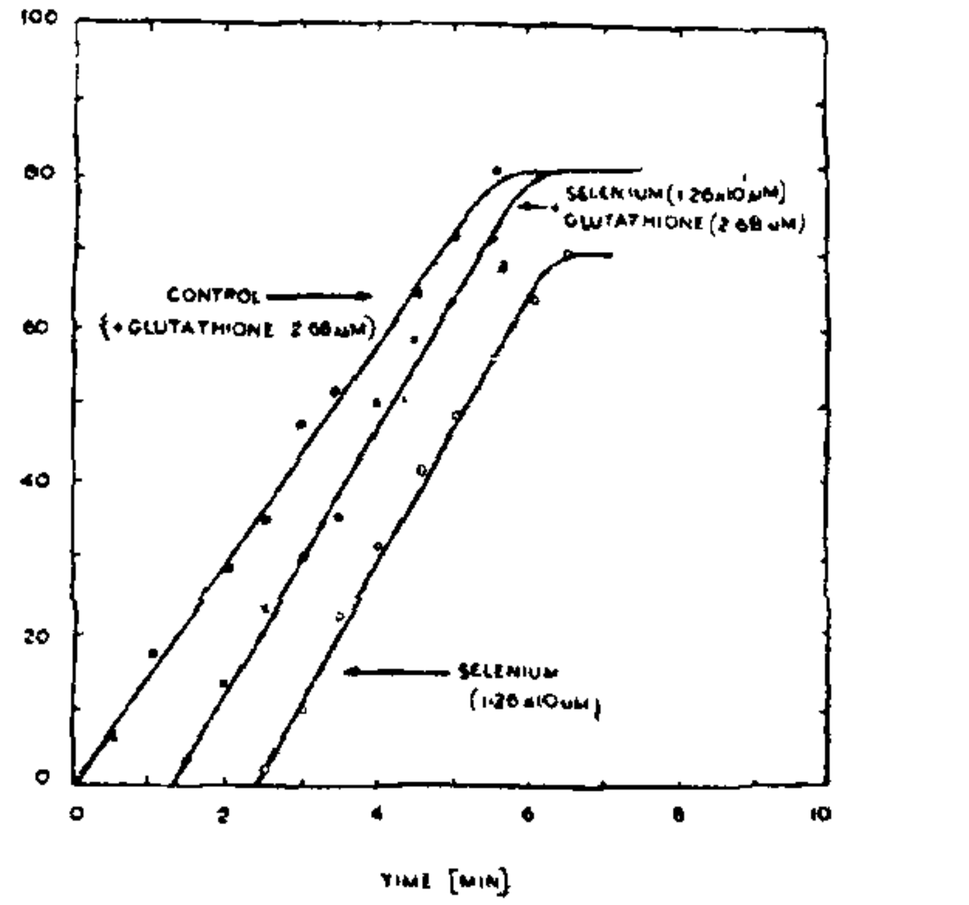

FIG. 2. Reactivation of selenium inhibited xanthine oxidase by glutathione. The reaction conditions were the same as described under Fig. 1. Selenium, as sodium selenite and glutathione were used in the concentration of $1{\cdot}26 \times 10^{-1}\ \mu$M and $2{\cdot}68\ \mu$M, respectively.

*In a low concentration of selenium ($1{\cdot}26 \times 10^{-1}\ \mu$M), the xanthine oxidase activity was initially significantly lowered, and on further incubation, this inhibition was observed to be gradually reversed. The nature of inhibition was found to be non-competitive. On

Thus, with the results of the above observations it is difficult to assess the role of selenium in arthritis and gout, although a powerful inhibitory action of selenium on the activity of xanthine oxidase has been detected.

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* One unit of xanthine oxidase activity is defined as that causing an increase in O.D. of ·001 per minute, at room temperature and pH 7·4, in the presence of hypoxanthine, 7·0 μg.

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NEWER BASIS OF DIFFERENTIATING *ALTERNARIA HELIANTHI* FROM *ALTERNARIA CHRYSANTHEMI*

Helminthosporium helianthi causing blight disease of sunflower was first described by Hansford[1] in 1943. Later in 1969 Tubaki and Nishihara[3] made a detailed study of this pathogen and concluded that the fungus should belong to the genus *Alternaria* because of the presence of both transverse and longitudinal septa in the porospore type of conidia, and accordingly proposed a new combination : *Alternaria helianthi* (Hansf.) Tubaki and Nishihara comb. nov. They further observed striking similarities between the conidia of *Alternaria helianthi* and *Alternaria chrysanthemi* Simmons and Crosier but they failed to point out any distinguishing morphological character to differentiate these two species from each other. However, in the studies carried out by the senior author[2], chains of conidia were observed in *A. helianthi* differentiating it from *A. chrysanthemi* which does not form conidia in chains.

Cross inoculation tests reported by Tubaki and Nishihara[3] revealed that *A. helianthi* was pathogenic both on *Helianthus annuus* and *Chrysanthemum* sp., while *A. chrysanthemi* infected only *Chrysanthemum* sp. and not *Helianthus annuus*. Cross inoculation tests and host range studies of the two species of *Alternaria* were accordingly undertaken to throw additional light, if any, on the diversity of their pathogenic ability.

Twenty plant species, *viz.*, *Artemisia scoparia*, *Bidens biternata*, *Blainvillea latifolia*, *Calendula officinalis*, *Carthamus tinctorius*, *Cichorium intybus*, *Chrysanthemum indicum*, *Dahlia rosea*, *Eclipta alba*, *Erigeron canadensis*, *Gaillardia pulchella*, *Helianthus annuus*, *H. tuberosus*, *Lactuca sativa*, *Launaea nudicaulis*, *Parthenium hysterophorus*, *Pluchea lanceolata*, *Tagetes patula*, *Xanthium strumarium* and *Zinnia elegans*, all belonging to the family Compositae, were inoculated in the glass house with conidial suspensions in water of *A. helianthi* and *A. chrysanthemi* separately. The results of infection tests showed that *Alternaria helianthi* could infect *Calendula officinalis* and *Helianthus tuberosus*, two ornamentals, and *Blainvillea latifolia* and *Eclipta alba*, two annual weeds, besides *Helianthus annuus*. *Alternaria chrysanthemi*, on the other hand, infected only *Chrysanthemum indicum*. The inability of *Alternaria helianthi* to infect *Chrysanthemum indicum*, observed in the present investigation, is contrary to the observations made by Tubaki and Nishihara[3]. This may be due to different species of *Chrysanthemum* taken by them in the cross inoculation test. The hosts—*Blainvillea latifolia*, *Eclipta alba*, *Calendula officinalis* and *Helianthus tuberosus* found susceptible to *Alternaria helianthi* and not to *Alternaria chrysanthemi* thus serve as indicator hosts for differentiating the two species of *Alternaria*.

Thus *Alternaria helianthi* and *A. chrysanthemi*, considered to be morphologically similar, but differing in cross inoculation tests, can also be distinguished from each other on the basis of conidial chains as reported in *A. helianthi* only, and on the basis of the indicator host reactions discussed above.

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