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# GAS CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ASSAY OF ALKANE THIOL S-METHYLTRANSFERASE

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## SUMMARY

A method is described for the quantitative assay of the methylation of alkanethiols from the methyl-donor S-adenosylmethionine, catalysed by the microsomal enzyme S-adenosyl-L-methionine:thiol S-methyltransferase (E.C. 2.1.1.9). The reaction is carried out in sealed vials, one fifth of whose volume is taken up by an aqueous phase containing the enzyme and reactants. The volatile substrates and products of the reaction, thiols and thioethers, respectively, are present in equilibrium both in the liquid and gas phases in the reaction vessels. Aliquots of the gas phase are removed at intervals in gas-tight syringes, and analysis is performed directly on a gas chromatograph fitted with a flame-ionization detector. The amounts of thiol and thioether detected are then related to the total amounts of substance in the reaction vessels from calibration measurements, so that the kinetics of the enzymatic process can be evaluated. This technique offers distinct advantages over previously reported methods, in that no radioactively labelled compounds are required. Furthermore, decreases in substrate and increases in product can be assayed simultaneously, and the methylation of a mixture of thiols can be monitored in a single set of analyses.

## INTRODUCTION

An enzyme system present in the microsomal fraction of mammalian liver homogenates which catalyses methyl transfer from S-adenosyl-L-methionine (SAM) to "non-physiological" thiol compounds was first described by Bremer and Greenberg[1]. The enzyme was partially purified and characterized by Borchardt and Cheng[2]. Both groups employed an assay based on the use of $^{14}C$ in the active methyl group of the donor molecule SAM, and quantified the amount of labelled thioether product formed after solvent extraction procedures. The significance of methylation of alkanethiols as a detoxification mechanism was pointed out by Holloway *et al.*[3], since the thioether products are considerably less toxic than their thiol counterparts[4], probably owing to the blocking of the highly reactive thiol group. In a previous report[3], we suggested the possibility of analysing the volatile thiols and thioethers involved in the reaction by direct gas chromatographic (GC) methods. The obvious advantage is that

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both substrate and product are quantified simultaneously. Moreover, the need for radioactively labelled reagents is removed, which is a considerable advantage when dealing with substances as volatile as the alkanethiols and thioethers. At that stage, our method was only useful for qualitative, or at best semi-quantitative, investigations. The present paper describes the extension of the GC method for the quantitative assay of these methylation reactions, and its application to the study of the simultaneous methylation of a mixture of alkanethiols, which has not been possible using other methods.

## MATERIALS AND METHODS

The alkanethiols were obtained from Ega-Chemie (Steinheim, G.F.R.) or Fluka Feinchemikalien (Neu-Ulm, G.F.R.) and were of the highest available commercial quality. The respective thioethers for use as reference substances are not available commercially (except for dimethyl sulphide) and were synthesized from the equivalent alkyl bromides by treatment with sodium methylthiolate in a two-phase reaction system[5]. S-Adenosyl-L-methionine was purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade, and were purchased from normal sources.

The enzyme preparations were derived from the microsomal fraction of mammalian liver homogenates, as described previously[3]. Protein determinations were carried out by the method of Lowry *et al.*[6] using bovine serum albumin as standard.

The reactions for the enzymatic methylations were carried out in 5-ml glass vials, fitted with PTFE-coated butyl-rubber septums ("Bördelkappen"; Machery, Nagel and Co., Düren, G.F.R.). The reaction mixture sealed into the vial consisted of 1 ml of an aqueous phase containing 100 m*M* Tris·HCl buffer, pH 8, 1 m*M* SAM and 1–4 mg $ml^{-1}$ of microsomal protein. The vials were incubated continuously at 25°C. The reaction was initiated by the introduction of 50 $\mu$l of thiol, dissolved in 1 *M* NaOH to the extent of 0.2–1% (v/v). The final pH of the reaction mixture was thus 8.5. The thiol was injected through the septum by means of a gas-tight syringe fitted with a valve (Pressure Lok®, capacity 100 $\mu$l; Precision Sampling Corp., Baton Rouge, LA, U.S.A.). A similar syringe was employed for sampling of the gas phase of the reaction vessel at regular intervals (generally hourly); 20 $\mu$l of the gas phase were drawn out for GC analysis.

The gas chromatograph employed was a Hewlett-Packard Model 7600A, fitted with a flame ionization detector. Results were quantified through an integrator (Hewlett-Packard No. 3370A) and recorded on a Hewlett-Packard No. 7126A pen recorder. Glass columns were used in the instrument, and had internal and external diameters of 2 and 6 mm, respectively. Various lengths were selected, according to the particular analysis to be performed. For shorter-chain alkanethiols, a column length of 8 ft. was suitable, but this had to be progressively reduced for thiols with higher boiling points and correspondingly longer retention times. For $C_{10}$ thiols, for example, a 2–4 ft. column was more suitable. The column packing was 80% solid-phase Chromosorb P, mesh 60–80, and 20% liquid-phase silicone gum rubber 6C, grade SE-30. Isothermal conditions were set on the instrument of 100, 150 and 250°C for column, injection block, and detector, respectively.

## RESULTS

The number of theoretical plates, $N_{th}$, of the column under the conditions stated in the Materials and Methods section was calculated from the following expression[7]:

$$N_{th} = 5.54(T_{m+s}/b_{0.5})^2$$

where $T_{m+s}$ is the gross (absolute) retention time of a particular peak, and $b_{0.5}$ is the width of that peak at half its height. Irrespective of the actual length of column employed, the number of theoretical plates was calculated to be $225 \pm 25$ plates/ft. of packed column.

The retention times registered for a large series of straight- and branched-chain alkanethiols and the methylthioether derivatives are listed in Table I. Under the conditions employed, the retention times of the methylthioethers relative to the

TABLE I

RETENTION TIMES FOR A SERIES OF ALKANETHIOLS AND THE RESPECTIVE METHYL THIOETHERS UNDER THE ANALYTICAL CONDITIONS GIVEN IN THE TEXT

The compounds are listed according to the alkane residue, in order of ascending retention times; the number in brackets following the alkane residue refers to the substituent position of the thiol or methyl thioether group. The data were obtained using a 2-ft. column (see Experimental section). All the thiols listed here were found to be methylated by the microsomal S-methyltransferase.

| *Alkane residue* | *Retention times (sec)* | |
|---|---|---|
| | *Thiol* | *Thioether* |
| Methane | 25– 27 | 44– 45 |
| Ethane | 40– 42 | 71– 73 |
| Propane-(2) | 52– 54 | 99– 101 |
| 2-Methylpropane-(2) | 65– 66 | 134– 135 |
| Propane-(1) | 69– 71 | 123– 125 |
| 1-Methylpropane-(1) | 96– 97 | 184– 185 |
| 2-Methylpropane-(1) | 101–103 | 177– 179 |
| Butane-(1) | 123–125 | 224– 226 |
| Pentane-(1) | 226–228 | 416– 419 |
| Hexane-(3) | 304–307 | 557– 559 |
| Hexane-(1) | 424–426 | 780– 784 |
| Heptane-(1) | 796–808 | 1467–1476 |

respective thiols were higher by a factor of $1.82 \pm 0.08$. The retention times increased with increasing boiling point in a regular manner, demonstrating the suitability of the conditions set on the gas chromatograph. The transmethylation from SAM was investigated for all the thiols listed in Table I. The kinetics of the system were monitored by decreasing substrate and increasing product peaks in the gas chromatograms. An example is shown for 1-butanethiol in Fig. 1. The thiol peak, at a retention time of *ca.* 124 sec, is seen to disappear with progressing time, whilst the product, 1-butyl methyl thioether, appears at a retention time of 225 sec.

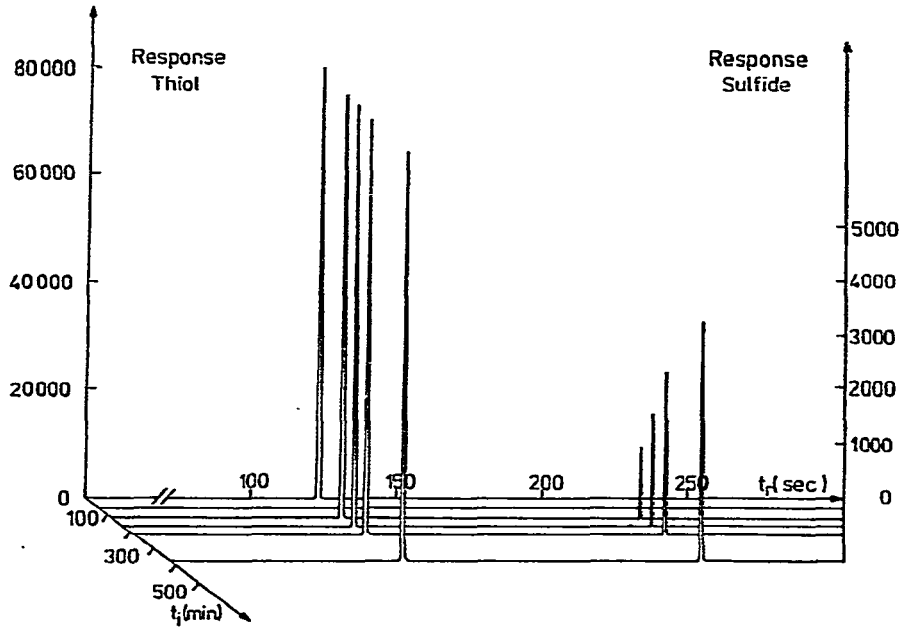


Fig. 1. The course of the enzymatic methylation of 1-butanethiol (retention time, $t_r$ = 124 sec). The product, 1-butyl methyl thioether, appears as a peak with $t_r$ = 225 sec. The reaction was monitored at regular intervals over a period, $t_t$, of 6 h, during which sufficient decrease in substrate and increase in product occurred to permit a quantitative assessment of the kinetics of the reaction (see Fig. 2). The signal responses for thiol and thioether are shown with different scales.

In principle, the enzyme activity can be expressed in terms of the rate of substrate disappearance or product appearance per unit time. In practice, however, such plots yielded unreliable results, since a major technical difficulty was encountered in the injection of exactly reproducible amounts of gas phase. Thus, a modified method was used to counteract this problem. Although the sample-to-sample amounts of substrate ($S_t$) and product ($P_t$) at given times varied owing to the injection inaccuracy, the relative amounts remained constant. Thus, a better expression of the increasing product was given by $P_t/(P_t + S_t)$. This quantity was then plotted as a function of time, $t$, as shown in Fig. 2. A linear increase was obtained over several hours of incubation. The slope of the plot, $m$, is then equal to $P_t/(P_t + S_t)t$. In the early stages of the process, $P_t \ll S_t$, so that $S_t$ approximates to $S_o$, the initial amount of thiol. Then, $m$ approximates to $P_t/S_o t$, whereby the product of $m$ and $S_o$ is $P_t/t$, equivalent to the activity of the enzyme. Experimentally, it turned out that this approximation held when the original amount of thiol in the 1-ml incubation volume was in the range 1–15 $\mu$mol. In the example given in Fig. 2, an initial amount of butanethiol of 9.33 $\mu$mol was employed. In fact, the final activity of the enzyme preparation was independent of the initial amount of thiol present within the concentration range give above, showing saturating conditions for the enzyme. The specific activity of methyltransferase in the microsomal fraction of pig liver homogenate which was employed in these experiments was 1.3 nmol $min^{-1}$ (mg protein)$^{-1}$ with butanethiol

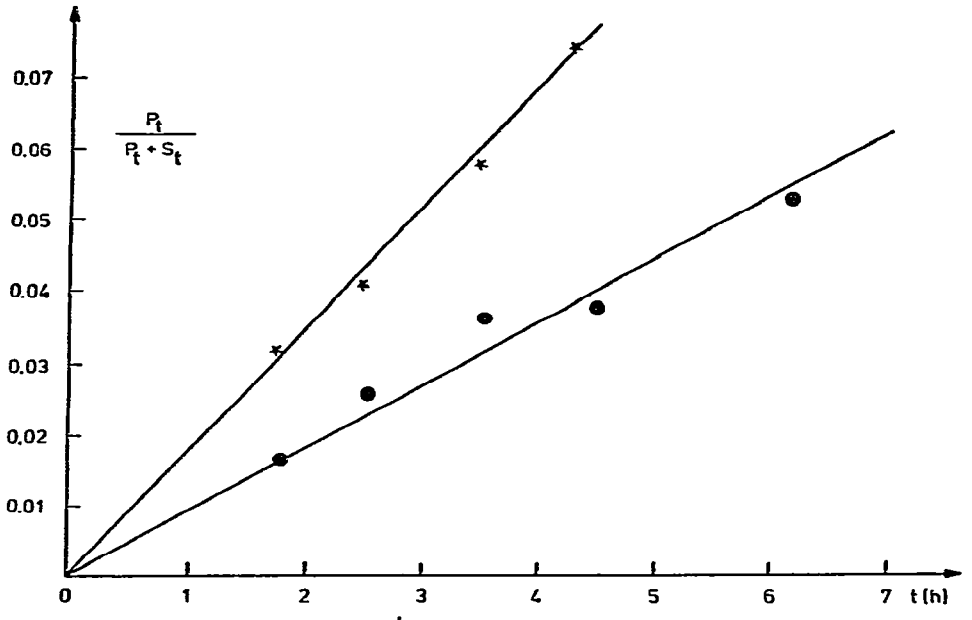


Fig. 2. Evaluation of the kinetics of methylation of 1-butanethiol (●) from the gas chromatograms shown in Fig. 1. The product formed, $P_t$, is expressed as a quotient of the sum of product and substrate, $P_t + S_t$, as explained in the text. The initial amount of 1-butanethiol in the incubation mixture was 9.33 μmol, and the enzyme employed, pig liver microsomes, yielded an activity of 1.3 nmol $min^{-1}$ (mg protein)$^{-1}$. Under identical conditions, ethanethiol (*) gave an activity of 3.9 nmol $min^{-1}$ (mg protein)$^{-1}$.

as methyl acceptor. For comparative purposes, ethanethiol under the same conditions gave a specific activity of 3.9 nmol $min^{-1}$ (mg protein)$^{-1}$.

Unfortunately, it is not possible to derive classical enzymatic constants such as $K_M$ values for this enzyme system directly, at least for the methyl acceptors, since the extremely low concentrations of thiol that would be required are impracticable from the analytical point of view. Thus, an alternative approach was adopted for examining the relative affinities of the enzyme for various substrates, namely by studying the simultaneous methylation of a mixture of thiols. An example is shown in Figs. 3 and 4 for an equimolar mixture (10.8 μmol each) of ethanethiol and 1-butanethiol. The substrate peaks are seen in Fig. 3 at retention times of 52 and 158 sec, respectively; ethyl methylthioether and 1-butyl methyl thioether appear at 91 and 300 sec, respectively. The plots of $P_t/(P_t + S_t)$ *vs.* $t$ shown in Fig. 4 yielded relative activities of 2.07:1 for 1-butanethiol and ethanethiol. In view of the fact that ethanethiol alone exhibited nearly three times the specific activity found with 1-butanethiol, this reversal in apparent activity with a mixture of the two thiols leads to the conclusion that the longer-chain thiol possesses a higher affinity for the enzyme, thus inhibiting the methylation of the ethanethiol.

## DISCUSSION

Alkanethiols are notoriously toxic compounds, whose metabolism and excretion must be ensured to prevent adverse effects on the organism. Methanethiol and

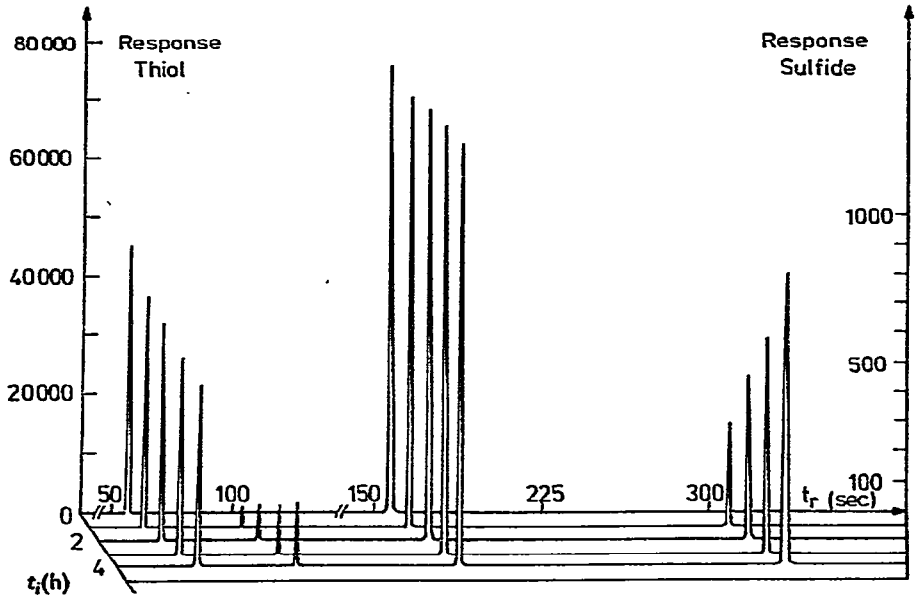


Fig. 3. The simultaneous methylation of a mixture of 1-butanethiol ($t_r$ = 158 sec) and ethanethiol ($t_r$ = 52 sec). The products, 1-butylmethyl thicether ($t_r$ = 300 sec) and ethyl methyl thioether ($t_r$ = 91 sec), are seen to increase during the 4-h ($t_i$) course of the reaction with decreasing thiol concentration. The retention times are not identical with those in Fig. 1 owing to the use of a different carrier-gas flow-rate. The time axis, $t_r$, is shown in two different scales, interrupted between the ethyl and butyl compounds to present a compact picture. The quantitative evaluation of the data is given in Fig. 4.

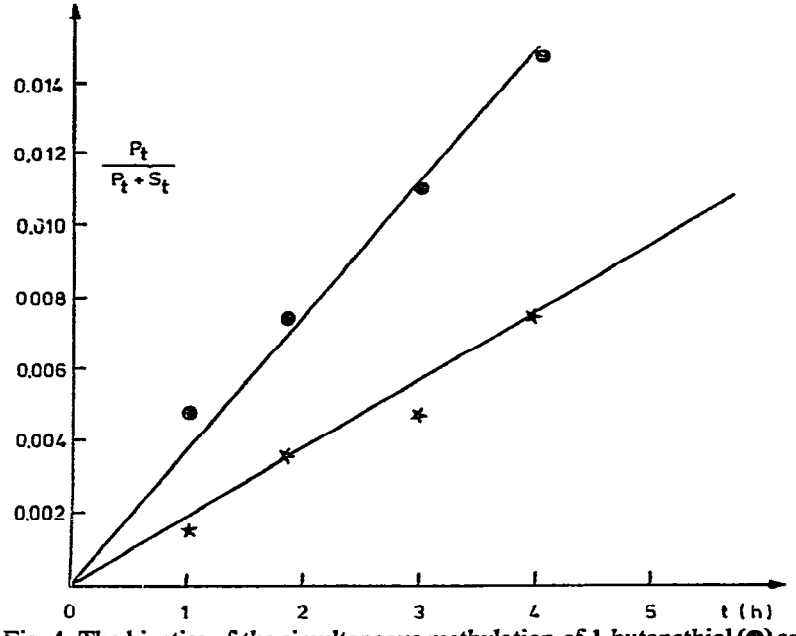


Fig. 4. The kinetics of the simultaneous methylation of 1-butanethiol (●) and ethanethiol (✶) derived from the gas chromatogram shown in Fig. 3. Each thiol was present in the reaction mixture to the extent of 10.8 $\mu$mol. The enzyme preparation employed methylated 1-butanethiol and methylated ethanethiol at activities of 0.347 and 0.175 nmol $min^{-1}$ (mg protein)$^{-1}$, respectively. Thus, the order of the relative activities of the two substrates is reversed compared with the methylation of each thiol on its own. For further discussion, see text.

ethanethiol are of endogenous importance, being produced by bacterial action in the gut[8], and possibly also in other tissues through enzymatic action[9]. Furthermore, thiols can be introduced into the system through foodstuffs, such as onions or garlic, and high levels of these toxins can be expected in the environment in heavyindustrial or volcanic regions. Although there is some information to suggest[10] that thiols are metabolized by full oxidation to $CO_2$ and $SO_4^{2-}$, there is ever-increasing evidence that methylation may play an even more important role in detoxification. For example, it has been observed that patients with severe liver diseases exhale large amounts of dimethyl sulphide, giving rise to the phenomenon of *foetor hepaticus*[11], although it is actually methanethiol which is the original component of the endogenous intoxication in such diseases. In order to be able to carry out some enzymological investigations of the enzyme systems responsible for methylation of alkanethiols[3,12], we have developed a relatively accurate analytical method using gas chromatography as described in this publication. In contrast to our previous report, this technique can be applied for quantitative assays of the enzyme. The primary advantage is that no radioactively labelled substances are required, as was the case with earlier reports[1,2], so that experiments can be carried out using volatile substrates such as the alkanethiols. A further advantage is that a multitude of thiols and thioethers can be analysed from a single gas chromatogram, so that simultaneous methylation of a mixture of thiols can be monitored. This was not possible with other techniques. The only major problem encountered with this method is the limitation imposed by the reproducibility when injecting gas phase into the instrument. Nonetheless, the accuracy is acceptable when one applies the method of evaluation described in the Results section, whereby product formed is expressed in terms of the total thiol and thioether amounts detected. The enzymological aspects of S-adenosyl-L-methionine:thiol S-methyl-transferase studied in our laboratories using this GC method will be reported in detail elsewhere.

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