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DEVELOPMENT AND COMPARISON OF THIN-LAYER CHROMATOGRAPHIC AND GAS–LIQUID CHROMATOGRAPHIC METHODS FOR MEASUREMENT OF METHIMAZOLE IN RAT URINE

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SUMMARY

Thin-layer chromatographic and gas–liquid chromatographic methods have been developed for the measurement of methimazole in rat urine. Of the two methods, gas–liquid chromatography is the more sensitive, but because of the instability of the derivative which is used, replicate measurements must be made on the same day. The densitometric method is less sensitive because of interference by endogenous matter, but is more rapid and hence better suited to routine use.

INTRODUCTION

Little is known of the metabolism in humans of the antithyroid drugs carbimazole and methimazole, but recent studies by ALEXANDER *et al.*[1], have shown that [^{35}S]carbimazole is metabolised with release of [^{35}S]sulphate. Carbimazole is also known to be readily hydrolysed in acid and alkaline media to methimazole, and is believed to owe its anti-thyroid activity to the formation of methimazole *in vivo*[2]. We have now confirmed that carbimazole is converted to methimazole in the presence of plasma *in vitro*. In seeking, therefore, to establish quantitative methods for determination of the distribution and fate of carbimazole when administered to human patients, we have focused attention firstly on methods for the detection and determination of methimazole.

Both infrared absorption[3] and titrimetric procedures[4,5] for the determination of methimazole are generally applicable only to relatively pure materials and could in no way be adapted to the very small quantity of methimazole expected in an extract of a biological fluid. The determination of thioimidazoles and thiopyrimidines colorimetrically using 2,6-dichloroquinonechlorimide[6,7] although potentially applicable to measurements on biological extracts has been applied only to relatively pure methimazole. The sensitivity of the reaction is about 10 μg and we have, therefore, examined the use of this reagent for the development of spots, and for the quantitative determination of methimazole in urine extracts after chromatography on thin-layer plates.

Gas–liquid chromatography (GLC) is often the method of choice for the determination of drugs in low concentrations in blood and urine. CLARKE[8] has reported that methimazole can be eluted from SE-30 columns, but this method proved unsatisfactory in our hands. We have found, also, that the methimazole peak shows considerable tailing on Apiezon L columns. We have, therefore, extended our studies of gas chromatographic methods to include alternative column packings, and methods based on the chemical modification of methimazole on the column.

EXPERIMENTAL

Materials and apparatus

Solvents. The solvents used were of A.R. grade or were re-distilled: methimazole (Nicholas Research); 2,6-dichloro-*p*-benzoquinone-4-chlorimine (DCQC), methyl iodide re-distilled, and *n*-tetradecane (B.D.H. Laboratory reagents).

Densitometric measurements. These were performed on a Chromoscan recording and integrating densitometer with a thin-layer attachment (Joyce, Loebl & Co. Ltd.). Operating conditions as follows. Chromoscan: aperture 10 × 0.5 mm, cam D, gain 5, optical wedge 0–0.5 O.D., light source 12 V, 100 W quartz iodine lamp. Thin-layer attachment: filter 465 mμ, aperture 10 × 1 mm, specimen expansion ratio 1:1, light source 12 V, 100 W standard tungsten projection lamp. The reflectance method of scanning was used.

Thin-layer chromatography. The plates (0.25 mm) were prepared from Merck Silica Gel G with zinc silicate (1%) as a phosphor. Plates (20 × 20 cm) were activated for 30 min at 110°, and stored at 30°. The developing solvent was the organic phase of a well-shaken mixture of chloroform–methanol–water (160:40:25), which gave R_F values of 0.44–0.46 for methimazole when freshly prepared. The detection reagent was 2,6-dichloroquinonechlorimide solution 0.4% in ethanol[6].

Gas–liquid chromatography. GLC was performed on a Perkin-Elmer F11 with glass columns (6 ft. × 4 mm I.D.) packed with either Carbowax 20M (10%) plus potassium hydroxide (5%) on Chromosorb W (100–120 mesh), hereafter called column A; or Apiezon L grease (10%) plus potassium hydroxide (5%) on Chromosorb W (100–120 mesh), hereafter called column B; column temperature 180°; nitrogen flow rate 31 ml/min.

Methods

Animals, dosing and extraction of methimazole. Six male Sprague-Dawley rats (approx. 250 g) were used. Five of the rats were injected intraperitoneally with methimazole (2.17 mg) in water (0.5 ml). The sixth rat was used as control. The animals were kept in separate metabolism cages and no restriction was placed on food and water supplies. Total urines were collected over a period of 12 h and each made up to 20 ml with de-ionised water. Diluted urine (9.8 ml) was transferred to a separator (100 ml), saturated with sodium chloride and extracted with chloroform (3 × 50 ml).

Densitometric determination. Standard solutions of methimazole in chloroform were made, such that a volume (20 μl) contained 0.3, 0.4, 0.5, 0.6 and 0.7 μg for application to the plate. Samples were applied with a microliter pipette (Marburg; 10 μl; Eppendorf, Hamburg, G.F.R.).

The chloroform extracts obtained (above) were adjusted to appropriate volumes,

so that 20–30 μl of the extracts contained a suitable amount of methimazole for application to the plate.

Five standards plus three samples of the chloroform extract were applied to the plate approximately 2 cm from the edge of the plate, 2 cm apart and with a resultant diameter of the spot no greater than 5 mm. Plates were developed, dried at room temperature, sprayed with McALLISTER's reagent, and allowed to stand for 10 min. The entire width of the spots was scanned perpendicular to the solvent flow. For reflectance scanning, a piece of thick white filter paper was placed underneath the plate[9].

Gas–liquid chromatographic determination. Column A: Methimazole (0.2, 0.4, 0.6, 0.8 mg) was dissolved in chloroform (1 ml). Each solution (1 μl) was injected onto the column, and after 5 min a subsequent injection (1 μl) of methyl iodide in acetone solution (0.1 ml/ml) made using a different syringe from that used for the methimazole injection. A calibration curve was plotted.

The peak of the derivative formed on column appeared at the retention time for S-methylmethimazole (3.2 min).

Column B: S-Methylmethimazole standards were prepared as follows. Methimazole (0.25, 0.5, 0.75, 1.0 mg) was dissolved in chloroform, methyl iodide (0.2 ml) added, and the solution left for 2 min. The volatile components were removed in a stream of nitrogen to dryness and methanol containing internal standard (*n*-tetradecane, 0.1 mg/ml) was added to each flask. Injections (1 μl) onto column B of each concentration were made to obtain the calibration curve.

Urine extracts. The chloroform extracts used for the densitometric determinations were evaporated to dryness in a stream of nitrogen, and methylated as de-

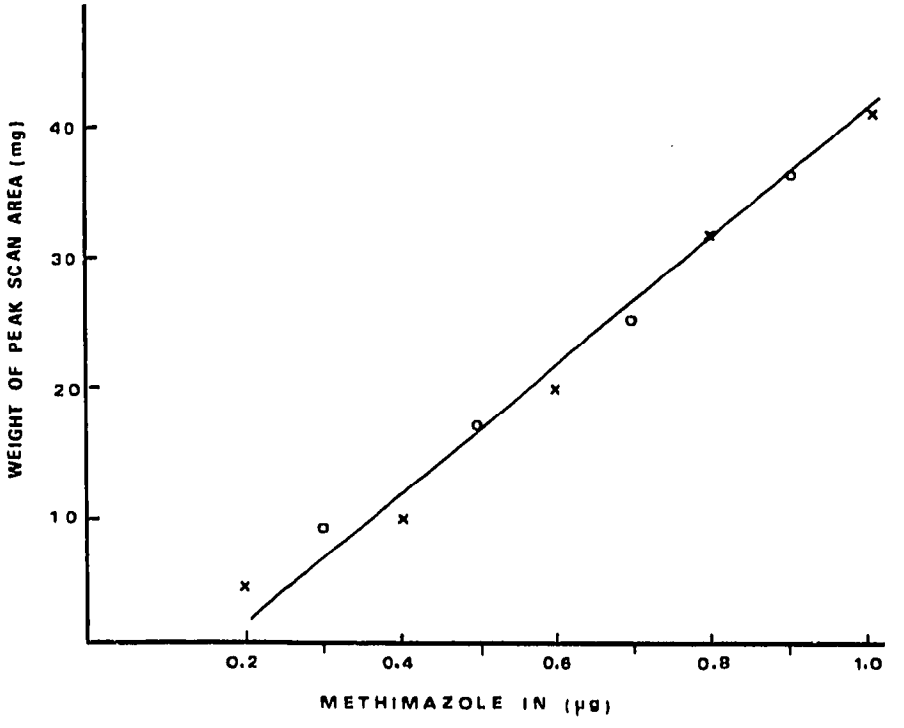

Fig. 1. Standard curve for pure methimazole added directly to plate (×—×—×) and recovered from urine (○—○—○).

scribed for the S-methylmethimazole standards. Methanol (100–200 μl according to expected drug concentration and containing *n*-tetradecane) was added to each residue, and injections (1 μl) made onto column B.

A chloroform extract of urine from the control was methylated by the same procedure, the residue was dissolved in methanol (100 μl) and examined by GLC. No peak appeared at the retention time for internal standard (5.4 min) or for S-methylmethimazole (2.0 min).

RESULTS AND DISCUSSION

Densitometric determination. A typical standard curve for pure methimazole in solution is shown in Fig. 1. Direct correlation between peak height and concentration was not possible. Other workers[9–11] used various methods of area measurement to correlate with the concentration of drug. We have used two methods (a) weight of peak scan area, and (b) area as defined by peak height × width at half altitude[9].

Table I shows the amounts of methimazole found in the urine extracts obtained using these methods, with a standard curve constructed for each plate. The overall

TABLE I

COMPARISON OF DENSITOMETRIC AND GAS–LIQUID CHROMATOGRAPHIC METHODS FOR THE MEASUREMENT OF METHIMAZOLE CONTENT OF DILUTED URINE SAMPLES

Rat	*Methimazole content (μg ± S.D.)*		
	Densitometric method		*GLC method*
	By area	*By weight*	
1	29 ± 2.2	30 ± 2.2	29 ± 1.2[a]
2	165 ± 5.4	168 ± 6.5	165 ± 5.6[b]
3	97 ± 5.1	98 ± 4.8	94 ± 2.4[b]
4	76 ± 3.6	76 ± 3.7	
5	154 ± 8.8	154 ± 8.4	153 ± 4.4[a]

[a] Calculated on the basis of six replicate experiments.
[b] Calculated on the basis of five replicate experiments.

mean and standard deviation are shown for the whole set of results. There is good agreement between the results obtained by weight and by area determination and, as there is no significant difference between the means (Student's *t* test), measurement of area was adopted for routine use since it is less time consuming.

For quantitative work with solutions of methimazole, McALLISTER[6] found that the sensitivity of the colour reaction was about 10 μg. By contrast the present densitometric method is capable of detecting 0.1 μg methimazole on the thin-layer plate. Further work[7] with DCQC showed that this reagent gave quantitative results with ether extracts of urine containing propylthiouracil but the lower limit of sensitivity was 20 μg. Thiourea, however, also reacts with DCQC[6]. It could, therefore, interfere in the colorimetric assay when drugs such as propylthiouracil and methylthiouracil are used, since it has been reported[12] that methylthiouracil breaks down to thiourea in the rat. The densitometric method, however, is free of interference by both break-

down products and endogenous materials, since 100% recovery was obtained in recovery experiments from urine (Fig. 1). The complex formed is yellow and is attributed to an S-substituted derivative[13]. Other reagents[14] were no more sensitive to methimazole than DCQC.

DALLAS[9], and SHELLARD AND ALAM[15] reported that the thickness of the layer on the plate, time of development and positioning of drug spot in the densitometer were important factors affecting the precision of densitometric methods. In our experience, calibration curves also showed variations from plate to plate substantiating that layer thickness affected reproducibility between plates. On the other hand, by virtue of the procedure adopted, time of development of the plate with respect to the nature of the solvent and distance travelled by the solvent were reasonably constant. The initial application of the sample was found to be fairly critical and the spot size was kept to 5 mm in diameter. This on development gave a symmetrical spot.

Conditions for measurement of the yellow coloured complex after uniform spraying were found to be important. The maximum yellow colour was obtained in 10 min against a white background. Although the background appeared to the naked eye to be satisfactory, it was found when scanning the plate that the pen did not always return to the original baseline. Thus, in all measurements the area of the peak was determined from its preceding baseline[15].

Gas–liquid chromatographic determination. Preliminary work showed that whereas methimazole is retained on column A, subsequent injection with methyl

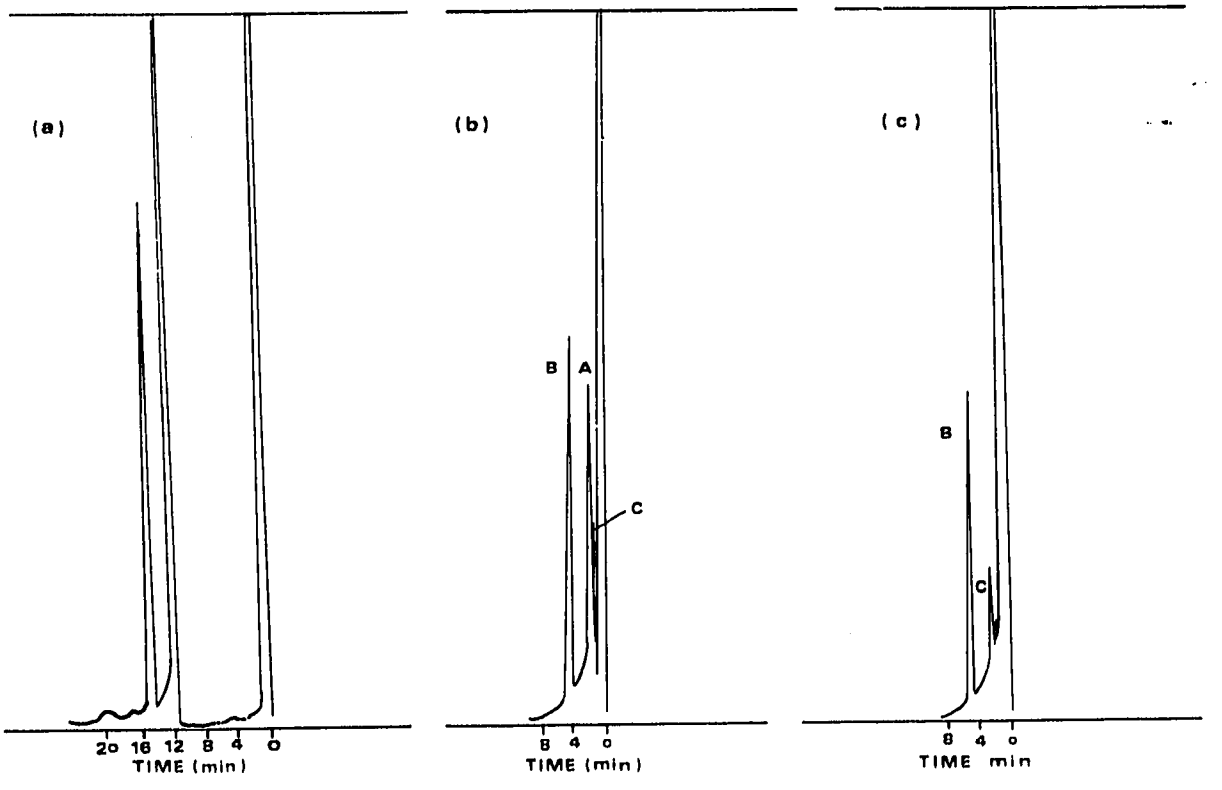

Fig. 2. (a) Methimazole in chloroform injected onto column A followed by methyl iodide at about 12 min. (b) Methimazole recovered from rats and converted to S-methylmethimazole (A); (B), internal standard and (C) endogenous material. (c) Control extract containing internal standard (B).

iodide in acetone gives a derivative peak (Fig. 2a). This was attributed to formation of the S-methyl derivative and confirmed when authentic S-methylmethimazole was injected on the same column and found to have the same retention time. A quantitative procedure was, therefore, developed using this observation, since most endogenous materials in biological extracts were removed from the column prior to derivative formation. Although a quantitative relationship could be obtained for on-column methylation of methimazole, it was not satisfactory with low concentrations in chloroform extracts of urine, since under these conditions a broad solvent peak often masked the S-methylmethimazole peak when methyl iodide was injected.

Column B was found to be satisfactory, and a linear relationship between the S-methylmethimazole peak height and concentration was obtained (Fig. 3). The derivative, however, was not formed *in situ* and it was necessary to pre-treat extracts with methyl iodide prior to injection. Column B gave the chromatogram for extracts containing drug and control as shown in Fig. 2b and 2c respectively.

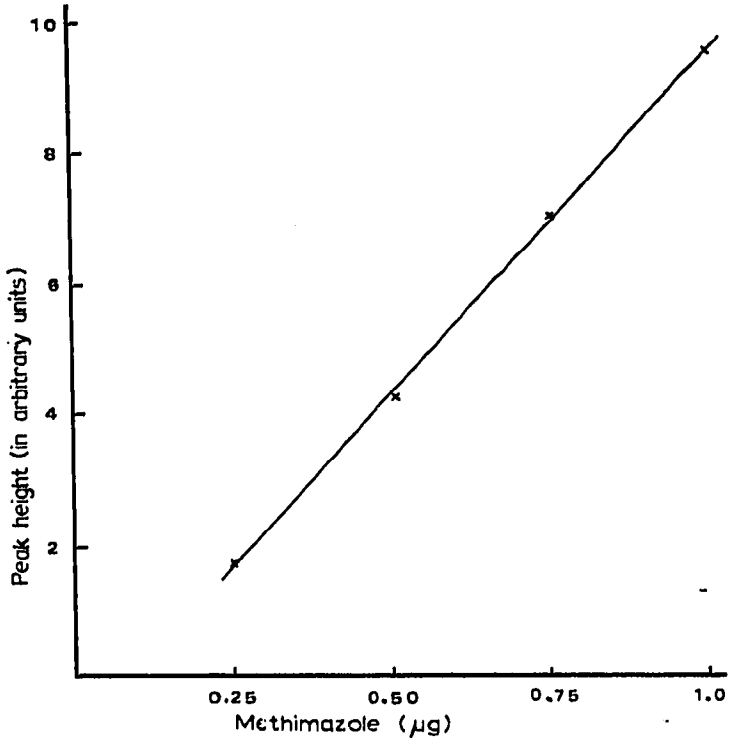

Fig. 3. Calibration curve for S-methylmethimazole by GLC.

Preliminary work with the methylation of methimazole showed that the reaction time was critical. A 10 min reaction time did not give as great a response as a 2 min reaction time and this was contrary to the observations of earlier workers. Solutions of S-methylmethimazole are, in fact, unstable if not stored below 0°. Thus, the whole procedure for a given urine extract was completed in one day.

Comparison of methods. Comparison of the densitometric and gas chromatographic results by Student's *t* test showed that there was no significant difference between the results of these two methods. The standard deviation, however, is less with the gas chromatographic method.

Although, therefore, the densitometric method has more variables than those

TABLE II

12 h EXCRETION OF METHIMAZOLE IN RAT URINE AS DETERMINED BY DENSITOMETRIC AND GAS–LIQUID CHROMATOGRAPHIC METHODS

Rat	*Percentage recovery of administered dose (i.p.)*	
	Densitometric method	*GLC method*
1	2.8	2.7
2	15.5	15.2
3	9.1	8.7
4	7.0	—
5	14.4	14.1

for the gas chromatographic method, it is more rapid and hence better suited to routine use. It is, however, less sensitive than the gas chromatographic method because of the possibility of overloading the plate with an excess of endogenous material when drug concentrations are low. The main disadvantage of the more sensitive gas chromatographic method is the instability of S-methylmethimazole, so that replicate measurements must always be made on the same day.

Table II shows the 12 h excretion of methimazole in the rat urine, expressed as a percentage of the dose administered (i.p.).

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