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A RAPID GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF THIAMPHENICOL IN BODY FLUIDS AND TISSUES

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SUMMARY

A gas chromatographic method for the qualitative and quantitative determination of thiamphenicol in plasma, urine and tissues has been developed. The procedure involves extraction of the drug from biological specimens with ethyl acetate, followed by drying and evaporation of the organic phase and separation of the trimethylsilyl derivatives with a gas–liquid chromatograph equipped with a flame ionization detector.

The method has been applied to samples from animals treated with 30 mg/kg of thiamphenicol by the intravenous, oral and intramuscular routes.

INTRODUCTION

Thiamphenicol (TAP), D(+)-*threo*-2-dichloroacetamido-1-(4-methylsulphonylphenyl)propane-1,3-diol, is a synthetic broad-spectrum antibiotic[1,2] in which the *para*-NO_2 group on the benzene ring of chloramphenicol (CAP) is replaced with a CH_3SO_2 group. TAP, unlike CAP, is not inactivated in the body; it is always present in the active form with particularly high and sustained levels in the liver–bile and kidney–urine[3–5]. It is therefore suitable for the treatment of infections of various etiology, either generalized or localized in different organs and systems. The literature concerning the clinical effectiveness of the drug is extensive[6–10] and information on the pharmacokinetics of TAP is also available[2,4,11–13].

For the determination of TAP in biological studies, the usual methods used are bacteriological assay[3,4] and the colorimetric method based on alkaline hydrolysis[14,15], but these methods have limited sensitivity.

The microbiological method cannot be used when other antibacterial agents are administered with thiamphenicol.

The colorimetric method is time-consuming and involves experimental difficulties; it is necessary to extract "free" thiamphenicol before the alkaline hydrolysis and to carry out successive colorimetric determinations of the aromatic aldehyde formed.

Aoyama and Iguchi[16] described a gas chromatographic method for the determination of TAP using an electron capture detector. Despite its high sensitivity, it is difficult to use this method for routine antibiotic determinations in biological samples.

In order to overcome these difficulties, a gas chromatographic method using a flame ionization detector has been developed that is simple and suitable for routine determinations in body fluids and tissues.

EXPERIMENTAL

Reagents

All reagents were of analytical grade.

The silylating reagent was Sil-Prep, obtained from Applied Science Laboratories Inc. Stock solutions were prepared by dissolving 1 g of thiamphenicol and of internal standard in 100 ml of methanol. The internal standard (I. S.) was D(+)-*threo*-1-(*p*-methylsulphonylphenyl)-2-acetamido-1,3-propane-diol. The thiamphenicol and the internal standard were products of Zambon S.p.A. Under refrigeration, these solutions were stable for several weeks.

Administration

The studies were carried out on male Wistar rats weighing 180–200 g. Thiamphenicol was administered by gastric tube or parenterally. The oral preparation consisted of a suspension in 2% gum arabic and the parenteral of an aqueous solution of the glycine ester, which is characterized by high solubility, rapid hydrolysis and complete absorption[17,18]. The rats were given 30 mg/kg of thiamphenicol as the free base in both instances.

Extraction procedure

A known amount of internal standard was added to 1–3 ml of the sample (plasma, urine and tissue homogenate) and the mixture extracted with 10 ml of ethyl acetate in a PTFE-lined screw-capped tube by shaking for 5 min.

After separation, the ethyl acetate phase was dried with anhydrous sodium sulphate and evaporated to dryness at 40° under a stream of nitrogen. The residue was dissolved in about 0.2 ml of methanol and transferred into a finely tapered 1-ml tube where it was again evaporated to dryness. The residue was then added to 10–50 μl of Sil-Prep and allowed to react for 15 min, after which time a 2–5 μl volume of the reaction mixture was injected in the gas chromatograph.

An emulsion was occasionally formed during the extraction (especially with tissue homogenates), and was broken by freezing in a dry ice–acetone bath, and the two phases were separated by centrifugation at 3,000 r.p.m. for 15 min.

Gas chromatography

A Hewlett-Packard 5750 gas chromatograph equipped with dual flame ionization detectors was used.

Column. A glass column, 10 ft. × 2 mm I.D., packed with 6.6% OV-3 and 3.3% OV-17 on Chromosorb W-AW DMCS, 70–80 mesh, was used. The column was conditioned at 290° for 3 days with a helium flow-rate of 15 ml/min.

Temperatures. The injection port was maintained at 280°, the column oven at 268° and the detector at 310°.

Flow-rates. The flow-rates used were: helium (carrier gas), 45 ml/min; air,

500 ml/min; hydrogen, 30 ml/min. The air and hydrogen flow-rates sometimes required slight adjustment in order to obtain maximal response.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, thiamphenicol has a retention time of approximately 10 min and the internal standard 6 min.

Fig. 1 shows the chromatogram of a standard solution of thiamphenicol and the internal standard in methanol.

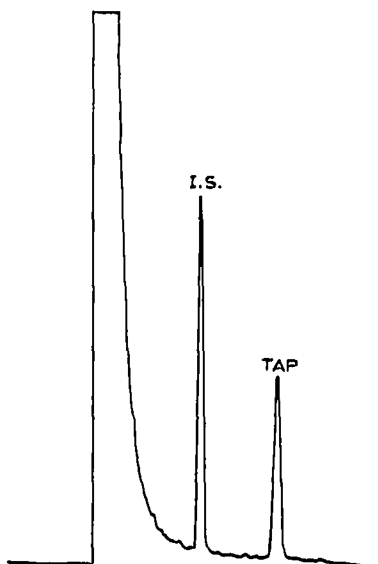
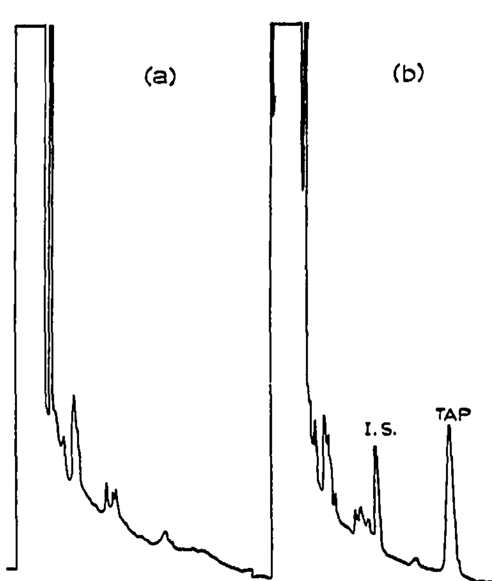


Fig. 1. GLC of standard solution of thiamphenicol and internal standard (TMS derivatives with Sil-Prep). Amount injected, 2 μg.

Figs. 2 and 3 represent chromatograms of an extract of 1 ml of plasma and urine blanks and of an extract of 1 ml of the same samples to which 9 μg of thiamphenicol and 6 μg of internal standard were added. No interfering peaks in the region of thiamphenicol and internal standard were observed in the chromatograms of several blank samples.

Data handling

After the chromatography, a baseline was drawn and peak heights were recorded for the TAP and internal standard peaks. The ratio of the peak height of TAP to that of the internal standard, corrected for attenuation if necessary, was calculated. Quantitation was carried out by referring to a standard curve constructed from values of known concentrations of TAP added to TAP-free samples. The ratio of peak heights was plotted against the concentration of TAP (μg/ml) and Fig. 4 shows that there is satisfactory linearity for peak ratios between 0.2 and 6. The internal standard should be added so as to obtain a peak ratio that lies between these limits.



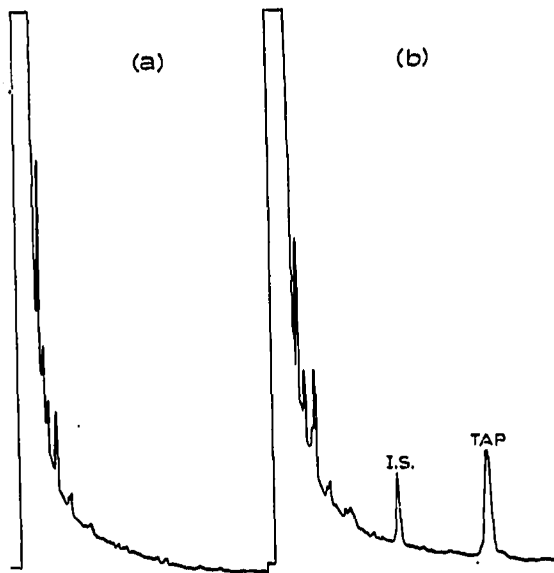


Fig. 2

Fig. 3

Fig. 2. GLC of (a) an extract of 1 ml of plasma blank and (b) an extract of 1 ml of the same sample to which 9 μg of thiamphenicol had been added.

Fig. 3. GLC of (a) an extract of 1 ml of urine blank and (b) an extract of 1 ml of the same sample to which 9 μg of thiamphenicol had been added.

Losses due to extraction or any other manipulation were automatically compensated for by the presence of the internal standard.

The ratio remained the same from one column preparation to another; only when a column started to deteriorate and absorption occurred on the support did

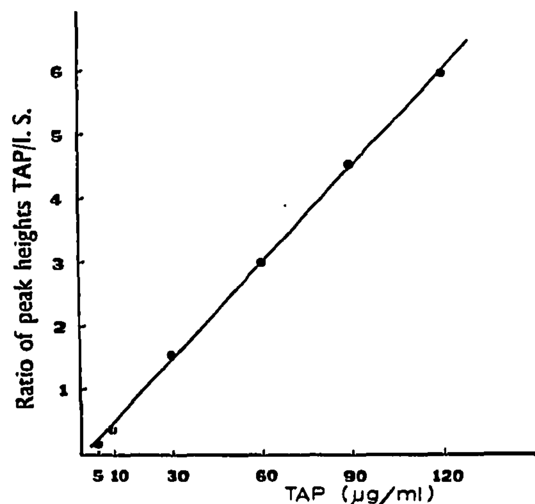


Fig. 4. Calibration curve for the determination of thiamphenicol in plasma and urine. Amount of internal standard added, 20 μg/ml.

TABLE I

DETERMINATION OF KNOWN AMOUNTS OF THIAMPHENICOL IN RAT URINE

Concentration (µg/ml)	*Assay values (µg/ml)*			*Difference (µg/ml)*		
	GLC	*Bacteriological*	*Colorimetric*	*GLC*	*Bacteriological*	*Colorimetric*
6.25	6.0	—	7.05	−0.25	—	+0.80
6.25	6.5	—	5.25	+0.25	—	−1.00
6.25	6.4	4.8	4.75	+0.15	−1.45	−1.50
12.5	12.3	8.0	9.8	−0.2	−4.5	−2.70
12.5	12.0	12.4	13.8	−0.5	−0.1	+1.3
12.5	12.6	7.15	16.3	+0.1	−5.35	+3.8
52.5	49.8	59.5	55.0	−2.7	+7.0	+2.5
52.5	54.0	49.5	47.5	+1.5	−3.0	−5.0
52.5	54.7	49.0	50.0	+2.2	−2.5	−3.5
105.0	109.0	120.0	110.0	+4.0	+15.0	+5.0
105.0	99.5	123.5	96.0	−5.5	+18.5	−9.0
105.0	101.5	105.8	101.2	−3.5	+0.8	−3.8

the ratio change. Our column has been in continuous use for 6 months without signs of deterioration. A mixture of TAP and internal standard is analysed daily; the peak heights are measured and the ratio compared in order to check the condition of the column.

Table I presents typical values for the determination of TAP in urine by the gas chromatographic, bacteriological and colorimetric methods. In this series of determinations, the t values for the differences between known and determined concentrations were 0.503, 2.931 and 0.943, respectively.

The storage of urine and plasma samples was examined. No significant changes took place in the urine and plasma results when stored for three months in the frozen state at −20°.

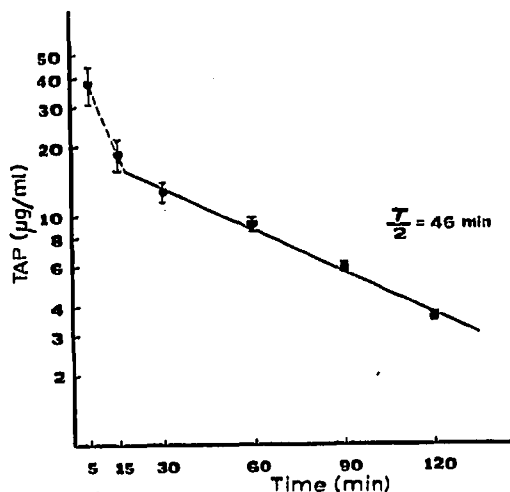


Fig. 5. Semi-logarithmic plot of plasma levels of thiamphenicol relative to time in the rat after treatment i.v. (30 mg/kg).

The procedure described was applied to samples of plasma from rats after a single dose of 30 mg/kg of TAP administered i.v., and 5, 15, 30, 60, 90 and 120 min after the dose of TAP, groups of five rats were exsanguinated. In the semi-logarithmic plot in Fig. 5, the rate of clearance of the antibiotic from the plasma is shown. The half-time of the drug is 46 min.

The urinary excretion of TAP in rats treated by the oral and intramuscular routes was also investigated. The rats, fasted for 18 h, were given a single dose of 30 mg/kg of TAP and were given water *ad libitum* and the urine from each rat was collected 4, 8, 24 and 48 h after treatment. The results obtained are given in Tables II and III. It is apparent that renal excretion is the principal mechanism of elimination of thiamphenicol from the body. The urinary excretion takes place completely in the active form, without preliminary biotransformation. In fact, the gas chromatographic–mass spectrometric analysis of the urine extract[10] has confirmed that the compound determined by this method is the antibiotic in the unmodified form (Fig. 6). The urinary recovery of TAP, as a percentage of the dose administered, varies between 50 and 80% for the oral route (Table II). Similar values were obtained after intramuscular administration (Table III).

This gas chromatographic method is more selective, rapid and sensitive than

TABLE II

URINARY RECOVERY OF THIAMPHENICOL IN RATS IN THE 48 h FOLLOWING ADMINISTRATION OF 30 mg/kg BY THE ORAL ROUTE

The results are mean values of five determinations.

	Interval of urine collection (h)	Volume collected (ml)	Unchanged TAP	
			Excreted (μg)	Recovery (%)
	0– 4	3.6 ± 0.7	1.048 ± 159	29
	4– 8	5.0 ± 0.4	970 ± 121	27
	8–24	21.0 ± 4.6	237 ± 42	7
	24–48	26.0 ± 8.3	Traces	
Total	0–48		2.255	63 (50–80)

TABLE III

URINARY RECOVERY OF THIAMPHENICOL IN RATS IN THE 48 h FOLLOWING ADMINISTRATION OF 30 mg/kg OF THE DRUG INTRAMUSCULARLY

The results are mean values of eight determinations.

	Interval of urine collection (h)	Volume collected (ml)	Unchanged TAP	
			Excreted (μg)	Recovery (%)
	0– 4	3.2 ± 0.6	1.240 ± 208	31.1
	4– 8	4.8 ± 0.7	478 ± 70	12.0
	8–24	13.7 ± 2.2	168 ± 17	4.2
	24–48	19.3 ± 3.0	Traces	
Total	0–48		1.886	47.3 (35–60)

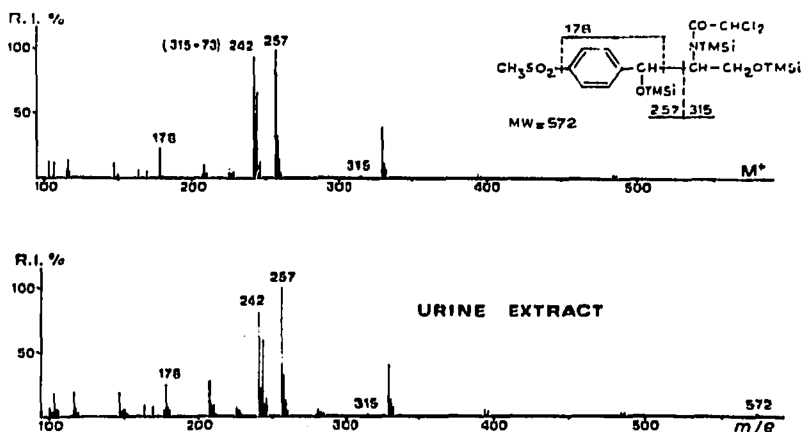


Fig. 6. Mass spectra of standard thiamphenicol and of a sample extracted from the urine of a rat treated with thiamphenicol.

the colorimetric and microbiological methods. The minimum amount of TAP that can be determined is about 0.2–0.4 μg/ml; this level has enabled the pharmacokinetics and metabolism of TAP in man and animal to be studied[20,21].

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