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GAS CHROMATOGRAPHIC DETERMINATION AND GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSIS OF CHLORAMPHENICOL, THIAMPHENICOL AND THEIR METABOLITES

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SUMMARY

A rapid and accurate gas chromatographic (GC) method is described for the determination of chloramphenicol, thiamphenicol and their metabolites excreted in human urine. These excretions were pre-treated with diazomethane and N,O-bis(trimethylsilyl)acetamide, so that they could be easily subjected to GC with satisfactory separation from each other and also from other urinary excretions, and could be determined simultaneously. The structures of the metabolites were confirmed by GC combined with mass spectrometric measurements of the GC peaks. The application of the method to urine specimens enabled more precise results for the amounts of metabolites present to be obtained; the excretion of thiamphenicol glucuronide was not observed.

INTRODUCTION

Many workers have investigated the metabolic fates of chloramphenicol (CP)[1–5] and thiamphenicol (TP)[6–8] which are used clinically as broad-spectrum antibiotics. In the earlier investigations, it was found that CP administered to humans is partly deacylated (CP-base) and mostly conjugated with glucuronic acid (CPG), and these metabolites are excreted in the urine together with smaller amounts of unchanged CP, while TP, unlike CP, is hardly inactivated in the body, so that only a small amount of deacylated TP (TP-base) is excreted in the urine together with much greater amounts of unchanged TP. It is doubtful, however, whether thiamphenicol glucuronide (TPG) is excreted in human urine, and this aspect is discussed later.

On the other hand, the analytical procedures that were used in the earlier investigations were based mainly on either biological or chemical assay. The bioassay, in general, has the disadvantage that when other antibacterial agents are also present it cannot be used to determine most of the metabolites as they are often inactivated. The chemical assays for excreted CP (TP) usually involve solvent extraction followed by colorimetric or spectrophotometric determination, and for the quantitation of their metabolites the difference between the amount of total CP (TP) and that of the

unchanged form is measured either by the above methods or by using gas chromatography (GC). The GC methods reported[9,10], however, did not afford the separation and simultaneous determination of CP (TP) and their metabolites, but only the determination of TP alone[10]. When such an indirect method is applied to the determination of a small amount of the metabolites (*e.g.*, TPG), the error is sometimes so large that the amount of the unchanged form may appear to exceed the total amount[9]. Therefore, the inconsistent results for the amounts of the metabolites that appeared in the earlier papers, especially for that of TPG in human urine[10,11], may depend on the inaccuracy of the analytical methods rather than on differences between the individual specimens.

In this paper, we describe attempts to develop a rapid and accurate GC method for the separation and determination of CP (TP) and their metabolites simultaneously. The proposed method has been applied to the analysis of some urine specimens. GC combined with mass spectrometry (MS) and peak assignments confirmed the chemical structures and the identification of the GC peaks.

EXPERIMENTAL

Reagents

Commercial CP was used after recrystallization from water. TP and the authentic samples of TP-base and TPG were gifts from Eisai Co. (Tokyo, Japan). The authentic CP-base was synthesized in our laboratory according to the following procedure. A 500-mg amount of CP dissolved in 10 ml of 0.1 *N* sodium hydroxide solution was heated for 10 min on a boiling water-bath and the hydrolyzed product was extracted twice with 10 ml of ethyl acetate. The extract was evaporated to dryness and submitted to a thin-layer chromatographic separation, using 10 parts of *n*-butanol saturated with water mixed with 1 part of acetic acid as developing solvent and Merck Kieselgel HF_{254} as the stationary phase. From the spot at $R_F = 0.66$ (detected by examination under ultraviolet light), crude CP base was extracted with methanol, then purified by recrystallization from ethyl acetate. The purity of the CP-base obtained was checked by GC and its structure was confirmed by infrared and GC–MS measurements. Authentic CPG was obtained from the urine of CP-dosed humans according to the method of Kamil *et al.*[12]. N,O-Bis(trimethylsilyl)acetamide (BSA), methanol and acetonitrile were used without further purification. Diazomethane was prepared freshly in the usual manner on each occasion.

Gas chromatographic conditions

A Shimadzu GC-5AF$_3$P gas chromatograph equipped with a flame ionization detector and a column temperature programmer was used. The column was glass, 1 m × 3 mm I.D., packed with 1.5% OV-17 on Chromosorb W AW DMCS, 60–80 mesh. The column temperature was maintained at 180° for the first 6 min, then increased to 260° at the rate of 5°/min. The injection port temperature was 280°. The carrier gas was nitrogen at a flow-rate of 65 ml/min, adjusted by means of a mass flow controller.

Mass spectrometric conditions

A Shimadzu LKB 9000 mass spectrometer was combined with the gas chromatograph. The following operating conditions were used: separator temperature,

Urine specimens
↓ Add I.S. solution
Evaporate to dryness at 37°
Residue
↓ Add 5 ml of CH_3OH
CH_3OH solution
↓ Pipette 1 ml
Add 3–5 ml of ether solution of CH_2N_2
Evaporate to dryness at 37°
Residue
↓ Add 0.5–1 ml of CH_3CN and 0.2 ml of BSA
Evaporate to dryness under stream of N_2
Residue
↓ Add 0.1–0.5 ml of CH_3CN
GC analysis

Fig. 1. Procedure for sample preparation.

290°; ionization source temperature, 270°; ionization energy, 70 eV; acceleration voltage, 3.5 kV; and trap current, 60 μA.

Sample preparation

Prior to GC determinations of unchanged CP (TP) and the metabolites, urine specimens were pre-treated by the procedure shown in Fig. 1. In this procedure, the volume of internal standard (I.S.) solution added was kept constant (1.0 ml) when constructing all calibration graphs (see below), but was changed appropriately (0.3–2.0 ml) when treating the urine specimens so that each substance to be determined could be calibrated within the range of the corresponding calibration graph.

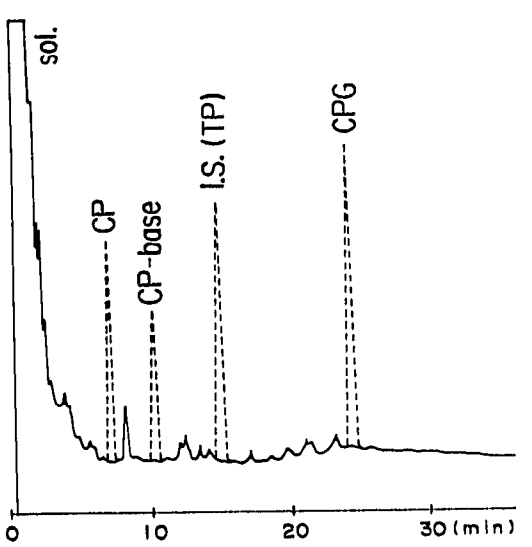
Calibration graph

CP was used as the internal standard for the determinations of TP and TP-base, and TP for those of CP, CP-base and CPG. The procedures for constructing the calibration graphs were similar in all instances. With CP, a known amount of CP dissolved in ordinary urine was treated by the procedure shown in Fig. 1, then submitted to GC under the above conditions. The peak height ratio of CP to TP (I.S.) was measured from the chromatogram and plotted against the weight ratio. The calibration graphs drawn in this manner through five points showed good linearity in all instances. Compared with the results obtained by direct methylation and formation of trimethylsilyl (TMS) derivatives (not dissolved in urine) followed by GC analysis, the recoveries of all the authentic materials treated by the procedure in Fig. 1 were found to be 98.6–101.0%. This indicates that the error in the present method is expected to be minimal.

RESULTS AND DISCUSSION

Gas chromatography

The peaks indicated by broken lines in Fig. 2 are due to authentic CP, CP-base,



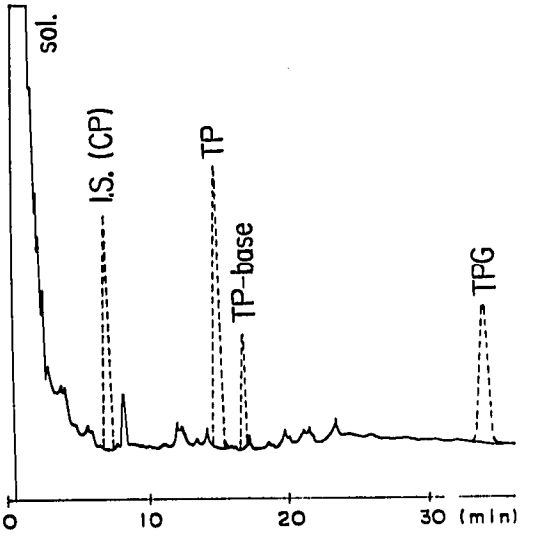
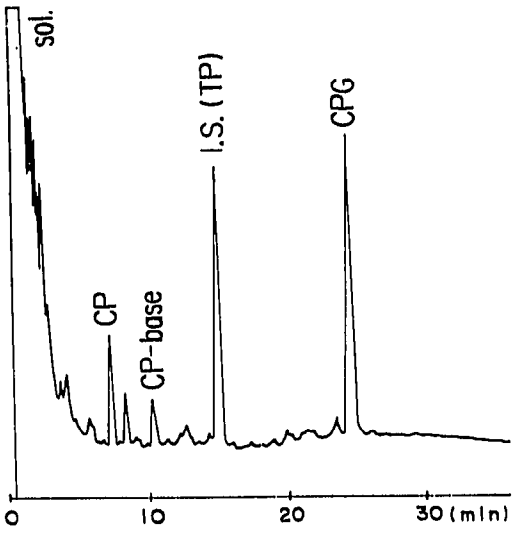


Fig. 2. Gas chromatogram of authentic CP, CP-base, CPG and background.

Fig. 3. Gas chromatogram of authentic TP, TP-base, TPG and background.

CPG and TP (I.S.) which were pre-treated with diazomethane and BSA. The solid line indicates a background chromatogram due to ordinary urine alone treated in the same manner. Fig. 3 shows the peaks of authentic TP, TP-base, TPG and CP (I.S.)



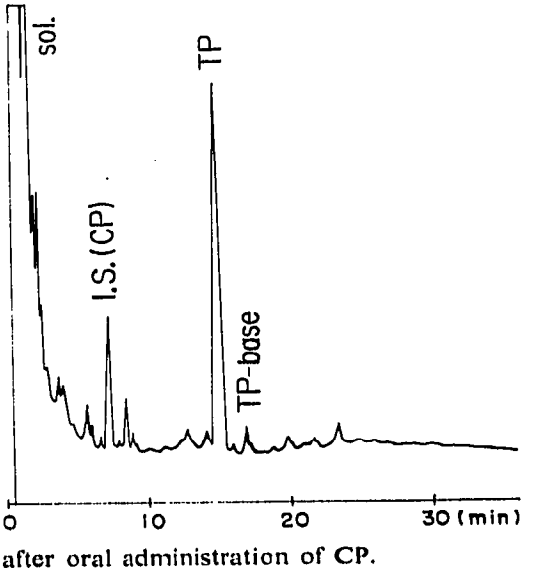


Fig. 4. Gas chromatogram of urinary excretions after oral administration of CP.

Fig. 5. Gas chromatogram of urinary excretions after oral administration of TP.

obtained in the same manner as those in Fig. 2. It is readily found from Figs. 2 and 3 that each authentic sample gives a single peak that is well separated from the others and also from the background peaks. These background peaks did not change their retention times in any of the ordinary urinary specimens tested. Figs. 4 and 5 show typical chromatograms due to urine specimens collected after oral administration of CP and TP, respectively. The retention times of the peaks in Fig. 4 agree well with the corresponding peaks in Fig. 2, and the same applies in Figs. 3 and 5. By detailed comparisons of Fig. 2 with Fig. 4 and of Fig. 3 with Fig. 5, it was found that no peaks due to unknown metabolites were detected in the urine of CP- or TP-dosed humans, and emergence of TPG was not observed. There have been a few papers reporting the excretion of very small amounts of TPG in human urine, but the quantitative results described in those papers seem to be inexact because they were obtained by such an indirect method that the amount of TPG was calculated from the slight difference between the amounts of unchanged TP and TP-base and that of total TP. Our results indicate that the amount of TPG, even if it may be excreted in human urine, is not greater than 0.05 ppm, which is the lowest limit of detection in the present method. These results confirm that CP, TP and their known metabolites other than TPG, excreted in human urine can be simultaneously determined by the present method.

GC–MS spectra

The GC peaks of authentic samples of CP, CP-base and CPG (Fig. 2), and TP, TP-base and TPG (Fig. 3) were submitted to MS measurements. The spectra obtained are shown in Figs. 6–11. These spectra, except for Fig. 11, agreed well with the corresponding peaks from urine specimens in Figs. 4 and 5. The peak assignments are summarized in the figures.

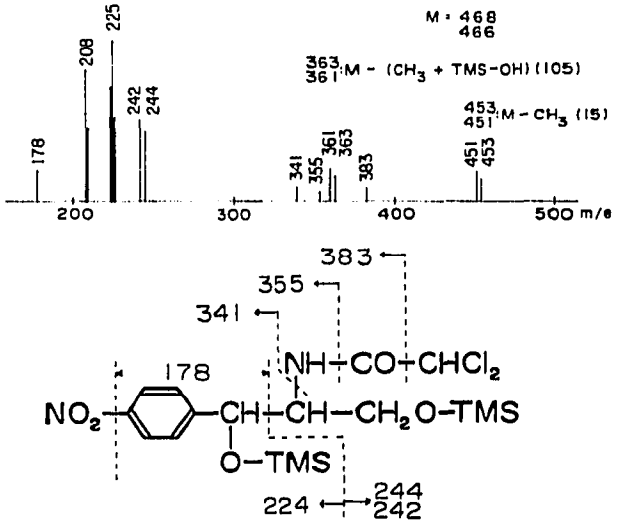


Fig. 6. GC–MS spectrum of CP.

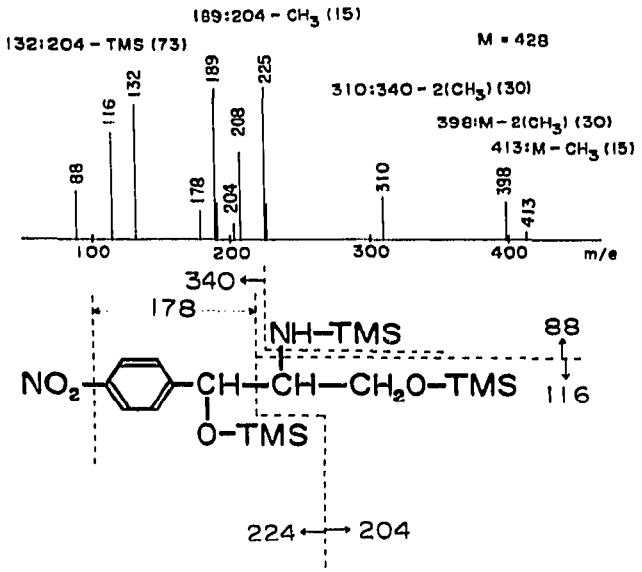


Fig. 7. GC–MS spectrum of CP-base.

The formation of the TMS derivative of CP was reported by Janssen and Vanderhaeghe[13], who stated that CP silylated with BSA in acetonitrile gave two distinct peaks due to bis- and tris-TMS-CPs. However, in our experiments, CP gave only a single GC peak due to bis-TMS-CP (see Fig. 2), and no other peaks were detected, even on standing for 48 h. The *m/e* values of the main peaks in Fig. 6 agree

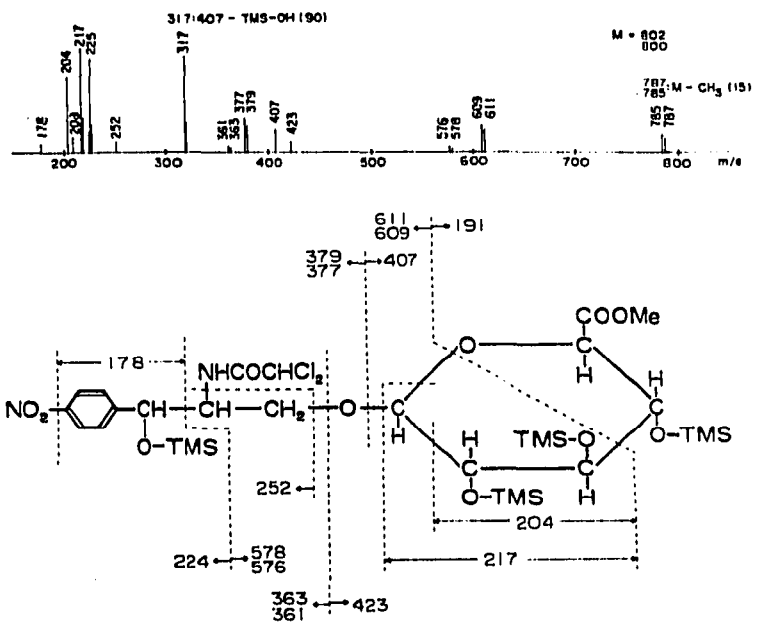


Fig. 8. GC–MS spectrum of CPG.

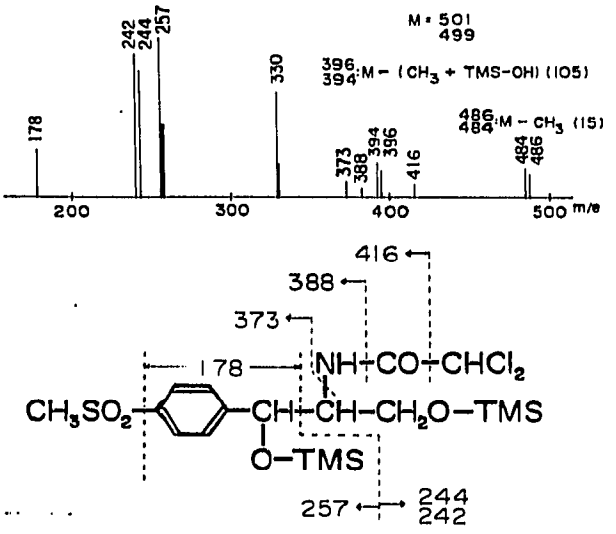


Fig. 9. GC–MS spectrum of TP.

well with those of bis-TMS-CP reported by Janssen and Vanderhaege[13]. From the spectrum in Fig. 7, it is considered that three TMS groups were substituted in a CP-base molecule by the procedure shown in Fig. 1, two being located at two hydroxyl groups and one at a deacylated amide function. An analogous situation applies to the spectrum of TP-base (Fig. 10). In the GC–MS spectrum of CPG shown in Fig. 8, the peaks at *m/e* 204, 217, 317, 407 and 423 are characteristic of methylated and TMS-substituted glucuronides. These peaks are also observed in the spectrum of authentic

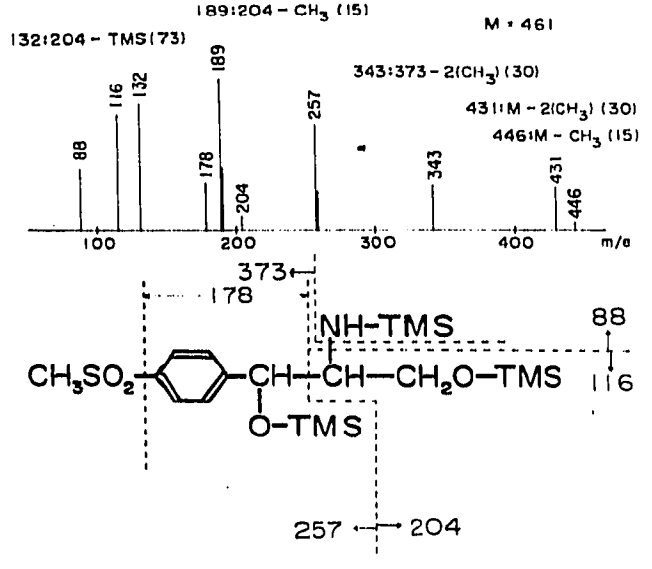


Fig. 10. GC–MS spectrum of TP-base.

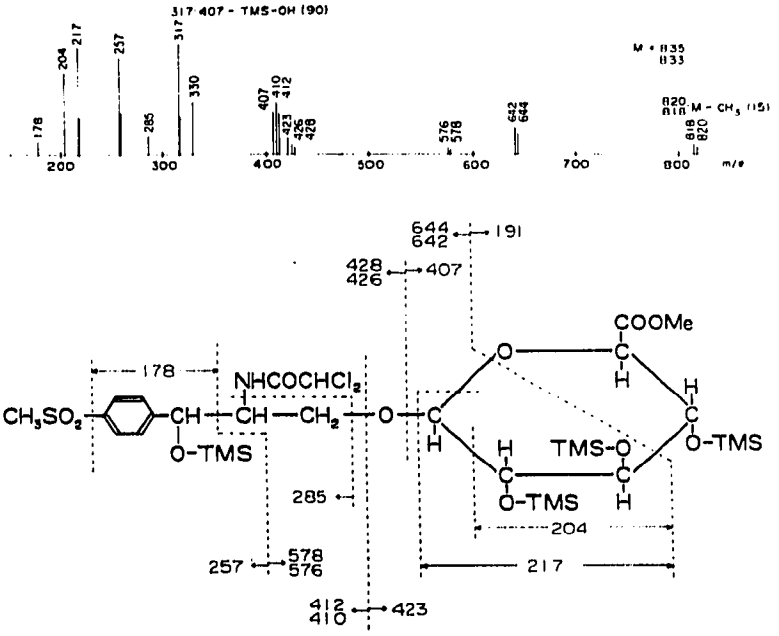


Fig. 11. GC–MS spectrum of TPG.

TABLE I

RECOVERY OF CP, CP-BASE AND CPG FROM HUMAN URINE AFTER ORAL ADMINISTRATION OF 500 mg OF CP

Time after administration (h)	*Volume of urine (ml)*	*CP (mg)*	*CP-base (mg)*	*CPG (mg)*	*Total (mg)*
0–2	146	7.29	4.33	48.34	59.96
	318	4.98	3.26	61.79	70.03
	98	8.83	4.95	57.65	71.43
	197	10.31	3.58	57.56	71.45
	104	7.78	7.27	42.20	57.25
		Average: 7.84 (1.57%)	4.68 (0.94%)	53.51 (10.70%)	66.02 (13.20%)
2–4	131	10.76	5.31	99.29	115.36
	88	6.71	5.60	79.99	92.30
	92	8.74	7.07	76.16	91.97
	154	10.64	3.94	86.11	100.69
	242	13.32	6.84	90.93	111.07
		Average: 10.03 (2.01%)	5.75 (1.15%)	86.50 (17.30%)	102.28 (20.46%)
4–6	137	7.21	4.66	55.72	67.59
	54	5.56	4.33	74.02	83.91
	106	6.52	6.92	58.55	71.99
	98	8.63	4.22	56.16	69.01
	102	9.70	9.00	57.65	76.35
		Average: 7.52 (1.50%)	5.83 (1.17%)	60.42 (12.08%)	73.77 (14.75%)
6–8	108	4.56	2.82	28.91	36.29
	76	3.39	4.98	54.44	62.81
	69	4.21	5.02	32.15	41.38
	70	5.49	4.50	39.46	49.45
	148	5.46	7.57	39.61	52.64
		Average: 4.62 (0.92%)	4.98 (1.00%)	38.91 (7.78%)	48.51 (9.70%)
		Overall average: 30.01 (6.00%)	21.24 (4.25%)	239.34 (47.87%)	290.58 (58.12%)

TPG (Fig. 11), indicating that CPG, as well as TPG, treated by the procedure shown in Fig. 1 underwent TMS-substitution at three hydroxyl groups in the glucuronic acid moiety and at an unconjugated hydroxyl group in the CP moiety, and was methylated at a glucuronic carboxyl group. Referring to the mass spectrum of the TMS derivative of TP reported by Gazzaniga *et al.*[10], it was expected that TP might undergo substitution of three TMS groups at two hydroxyl groups and an amide nitrogen atom. The spectrum in Fig. 9, however, indicates that only two hydroxyl groups in a TP molecule were substituted with TMS. This may be due to the difference in the method of silylation. In order to confirm the absence of TPG from the urine of TP-dosed humans, the GC–MS spectrum of the background chromatogram (Fig. 5) with the same retention time as that of authentic TPG (33.6 min) was measured. The resulting spectrum was unlike Fig. 11.

TABLE II

RECOVERY OF TP AND TP-BASE FROM HUMAN URINE AFTER ORAL ADMINISTRATION OF 500 mg OF TP

Time after administration (h)	*Volume of urine (ml)*	*TP (mg)*	*TP-base (mg)*	*TPG (mg)*	*Total (mg)*
0–2	198	68.17	1.53	—	69.70
	91	56.93	1.40		58.33
	138	64.93	1.77		66.70
	152	47.95	1.77		49.72
	151	56.48	1.55		58.03
		Average: 58.89 (11.78%)	1.60 (0.32%)		60.49 (12.10%)
2–4	109	101.15	1.62	—	102.77
	135	94.23	1.93		96.16
	108	85.35	2.09		87.44
	85	98.95	1.85		100.80
	124	110.89	1.90		112.79
		Average: 98.11 (19.62%)	1.88 (0.38%)		99.99 (20.00%)
4–6	146	64.73	1.49	—	66.22
	69	51.18	1.20		52.38
	102	69.35	1.46		70.81
	83	59.24	1.49		60.73
	73	72.63	1.41		74.04
		Average: 63.43 (12.69%)	1.41 (0.28%)		64.84 (12.97%)
6–8	96	36.40	0.79	—	37.19
	332	34.96	0.98		35.94
	137	44.72	0.94		45.66
	143	36.97	1.02		37.99
	252	45.82	1.04		46.86
		Average: 39.77 (7.95%)	0.95 (0.19%)		40.72 (8.14%)
		Overall average: 260.20 (52.04%)	5.84 (1.17%)	—	266.04 (53.21%)

Determinations of urinary excretions

The present method was applied to the determination of CP, TP and their metabolites excreted in human urine. The urine specimens were collected from five healthy adults every 2 h after oral administration of 500 mg of CP or TP. The results are shown in Tables I and II, where the values for the metabolites are given as CP and TP equivalents. These results show that the most remarkable difference in the excretion of CP and TP is in the amounts of their metabolites recovered; during 8 h after a single dose of CP, 82.4% of the total amount recovered in urine is excreted as the glucuronide and 7.3% as the deacylated form, while with TP, TP-base is the only metabolite, the amount of which accounts for about 2.2% of total amount of TP recovered. These results are similar to but more specific than those obtained by conventional methods. With minor modifications to the procedure, the present method enables CP, TP and their metabolites in other biological fluids to be determined. In those instances, it is advantageous to simplify the pre-treatment procedure as much as possible by utilizing the high resolving power of GC.

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