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Transformation and Excretion of Drugs in Biological Systems. III.[1] Separatory Determination of Metoclopramide and Its N^4-Glucuronide and N^4-Sulfonate in Rabbit Urine and Bile[2]

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The method of separatory determination of metoclopramide and its N^4-glucuronide and N^4-sulfonate, which are the major excrements in the urine and the bile of rabbits receiving metoclopramide, was established. Metoclopramide in the sample solution was extracted with chloroform under the condition of alkaline pH, and back-extracted with 1/15M KH_2PO_4 (pH 4.5). Metoclopramide in the aqueous layer was diazo-coupled in a brown test-tube which was used with a view to taking away the influence of light; thus, a developed color was measured at 540 mμ. On the other hand, the separation of the glucuronide and the sulfonate from each other was carried out by utilizing the difference in stability between them, which are hydrolysed in an acid medium. Resulting metoclopramide was isolated and determined as above-mentioned. This method was applied to the urine and the bile of rabbits administered metoclopramide intravenously.

Previously,[1] the six excrements in the urine of rabbits receiving metoclopramide were elucidated, and the major ones of them were found on the thin-layer chromatogram of metoclopramide-urine to be the unchanged form and its N^4-glucuronide and N^4-sulfonate. In this paper, in order to clarify the quantitative relationship among these major excrements, the method of separatory determination for them in the urine is proposed.

Pitel and Luce[4] carried out the determination of metoclopramide by the method depending on diazo-coupling with the aromatic amine; their attention, however, was paid only to the drug unchanged. In addition, since their procedure appears to be affected by light, a modification of the method is developed. The work on the separatory determination for N-glucuronide and N-sulfonate of aromatic amines has not been extended beyond the case of a sulfa drug[5] performed by means of paper chromatography. While, the method for metoclopramide presented here is a relatively simple and precise one utilized the difference in stability between the two conjugates in acid medium. An attempt, moreover, is made to apply this method to the bile specimens after administration of metoclopramide with the intention of exploring the possibility of entero-hepatic circulation for this antiemetic agent.

Experimental

Materials——Metoclopramide and its N^4-glucuronide and N^4-sulfonate were prepared as described previously.[1]

Reagents——All the reagents used in the present experiment were of special grade.

Apparatus——A Hitachi Perkin Elmer model 139 spectrophotometer was used.

Animals——Male albino rabbits weighing 2.5 to 3.5 kg were used.

1) Part II: T. Arita, R. Hori, K. Ito, K. Ichikawa, and T. Uesugi, *Chem. Pharm. Bull.* (Tokyo), **18**, 1663 (1970).
2) This work was presented at the 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1968.
3) Location: *Kita-12, Nishi-6, Sapporo.*
4) G. Pitel and T. Luce, *Ann. Pharm. Franc.*, **23**, 673 (1965).
5) T. Uno and Y. Sekiya, *Chem. Pharm. Bull.* (Tokyo), **14**, 687 (1966).

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Collection of Urine and Bile——Rabbits were anesthetized with pentobarbital sodium (25 mg/kg body wt.), and a polyethylene tube with external diameter of 2 mm for bile collection was inserted into the biliary duct by surgical operation. After 40 mg of metoclopramide dissolved in 10 ml of 0.9% NaCl containing 1.4 ml of 0.1N HCl was administered intravenously, bile specimens were collected at various times during the experiment and at the same time urine collections were made through Nelaton's catheter inserted to the bladder. The pH of urine was from 6 to 8 and that of bile about 8. These biological samples collected were analysed immediately.

Result and Discussion

Isolation of Metoclopramide from Sample Solution

The separation of metoclopramide from the N^4-conjugates, namely, the glucuronide and the sulfonate which co-exist in a sample solution was accomplished by the following method. To 1 ml of sample containing about 20 to 150 μg of metoclopramide, 5 ml of 0.1N NaOH and 5 ml of chloroform were added, and the mixture was vigorously shaken for 20 minutes and centrifuged. Three milliliters of the organic layer was then added to 4 ml of 1/15M KH_2PO_4 (pH 4.5) and the mixture was shaken for 10 minutes and centrifuged. Metoclopramide thus back-extracted into the aqueous layer was determined spectrophotometrically after diazotizaption and couling by the following manner.

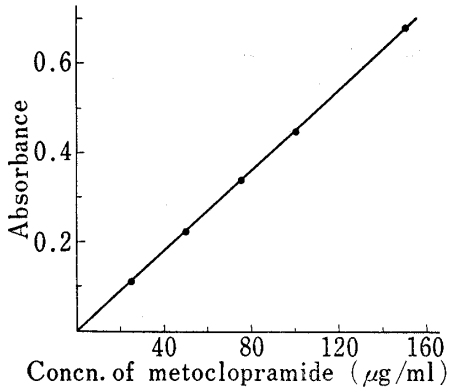

Fig. 1. Calibration Curve for Metoclopramide

Absorbance at 540 mμ measured after isolating and diazo-coupling was plotted against the concentration of metoclopramide in original sample solution.

Colorimetric Determination of Metoclopramide

A two ml aliquot of the aqueous layer was pipetted into a brown test-tube containing 5 ml of 2N HCl: The brown test-bute was adopted with a view to taking away the influence of light observed by using an usual colorless one. Under the condition of 0°, 0.5 ml of 0.1% $NaNO_2$ was added and the mixture was allowed to stand for 4 minutes. One milliliter of 0.5% ammonium sulfamate was then added. After 4 minutes, 1 ml of 0.1% N,N-diethyl-N'-(1-naphthyl)ethylenediamine oxalate was added. After the color was allowed to develop for 10 minutes, the absorbance was measured at 540 mμ within 60 minutes. The concentration of metoclopramide in an original sample is read from the calibration curve shown in Fig. 1.

Hydrolysis of Metoclopramide-N^4-glucuronide and Metoclopramide-N^4-sulfonate

As has been observed previously,[1] both these N^4-conjugates are stable at the alkaline pH; however, in the acid medium they may be hydrolysed,[6] though there is a difference in stability between them, giving rise to the parent compound. On the basis of such a fact, an attempt was made to find the conditions under which only the glucuronide is hydrolysed, but the sulfonate is not. At a temperature of 0°, 2 ml of 1N HCl was added to 1 ml of sample containing the glucuronide to be hydrolysed. After standing at this temperature for various times, the hydrolysis reaction was stopped by the addition of 3 ml of 1N NaOH, and metoclopramide produced was isolated and determined with the same procedure as described above. The result is shown with the hydrolysed ratio in Fig. 2. Thus, a constant value which is observed between 45 and 90 minutes after the start of reaction is corresponding to 98% of the used amount of the glucuronide. Although the sulfonate was also treated under the same conditions, the hydrolysis of this conjugate was found to be negligible. These

6) The stability of the glucuronide in various pH solutions will be described in the next paper.

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facts are greatly favorable to determine separately each of the glucuronide and the sulfonate. On the other hand, in cases in which the mixture of 1 ml of sample containing the sulfonate and 2 ml of 1N HCl was heated at a temperature of 100°, a constant value which shows the complete hydrolysis of the sulfonate was obtained between 2 and 6 minutes after the start of reaction as shown in Fig. 3. Incidentally, it was checked that no rupture of the acid amido bond of metoclopramide was observed under the above conditions.

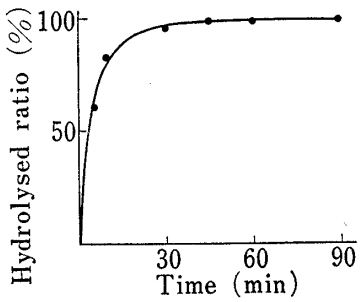

Fig. 2. Hydrolysis of Metoclopramide-N^4-glucuronide at 0°

abscissa: the time elapsed after the addition of 2 ml of 1N HCl to 1 ml of sample containing 200 μg of the glucuronide, at which the hydrolysis reaction was stopped by alkalizing the solution; *ordinate*: (amout of the glucuronide hydrolysed/amount of the glucuronide used) × 100

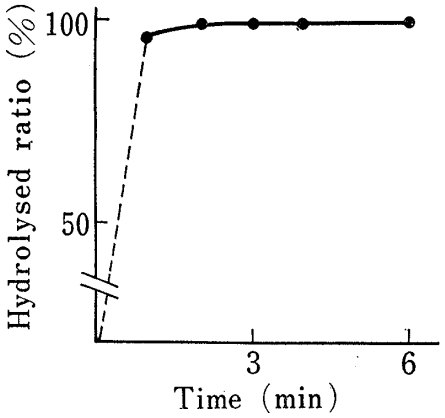

Fig. 3. Hydrolysis of Metoclopramide-N^4-sulfonate at 100°

abscissa: the time elapsed aftetr the addition of 2 ml of 1N HCl to 1 ml of sample containing 200 μg of the sulfonate, at which the hydrolysis reaction was stopped by alkalizing the solution; *ordinate*: (amount of the sulfonate hydrolysed/amount of the sulfonate used) × 100

Method of Separatory Determination

On the basis of these results, the separatory determination for metoclopramide and its N^4-glucuronide and N^4-sulfonate is carried out by the procedure shown in Chart 1. In each of a series of three test-tubes, a 1 ml aliquot of sample, if necessary, diluted adequately was placed. The aliquot in the first tube was treated to measure separately the concentration c_1 of the unchanged metoclopramide alone in the sample by the procedure mentioned in

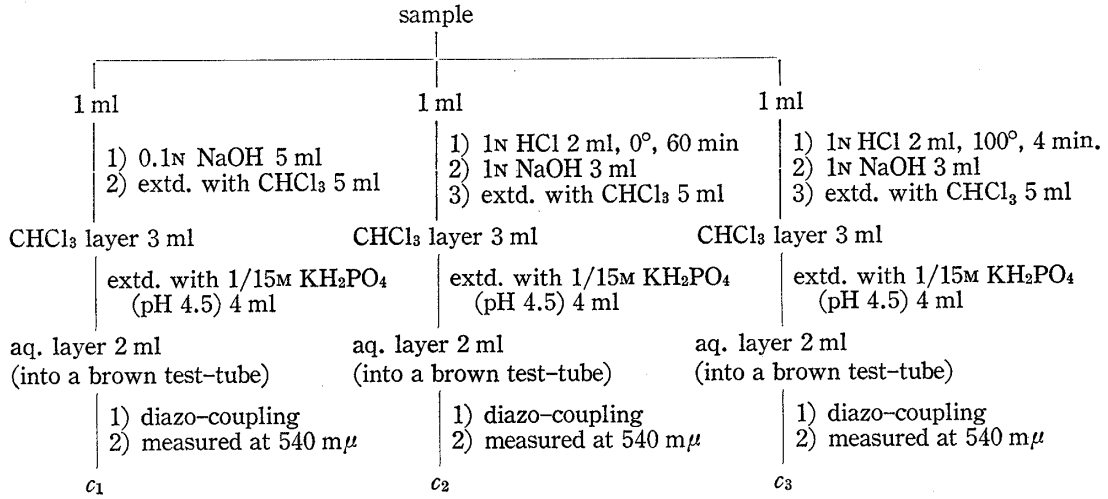

Chart 1. Method of Separatory Determination of Metoclopramide and Its N^4-glucuronide and N^4-sulfonate

c_1 : amt. of unchanged metoclopramide in 1 ml of sample
c_2-c_1: amt. of metoclopramide equivalent to the glucuronide in 1 ml of sample
c_3-c_2: amt. of metoclopramide equivalent to the sulfonate in 1 ml of sample

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"isolation and colorimetric determination of metoclopramide." Under the condition of 0°, on the other hand, 2 ml of 1N HCl was added to the second aliquot, and the mixture was allowed to stand for 60 minutes, by which the glucuronide alone is hydrolysed to produce metoclopramide being the parent compound. The reaction was then stopped by adding 3 ml of 1N NaOH; thus, the concentration c_2 of metoclopramide which had existed in the unchanged form and the glucuronide one in the original sample is determined. The last 1 ml was heated for 4 minutes at temperature of 100° after adding 2 ml of 1N HCl to hydrolyse the sulfonate also. The reaction mixture was then alkalized with 1N NaOH; thus, the total concentration c_3 of the three substances in question is obtained. Consequently, the substraction of c_1 from c_2 gives the concentration of the glucuronide and that of c_2 from c_3 the concentration of the sulfonate as metoclopramide equivalent. While it was demonstrated by the blank test that normal constituents of rabbit urine and bile do not interfere the above determination. In addition, after the rabbit urine and bile receiving metoclopramide were extracted with chloroform under the condition of pH 11—13, the chloroform extracts were chromatographed on the thin-layer plate of silica gel. As a result of the application of the above colorimetric procedure for each excrement[1] thus isolated, it was observed that the optical density for the deethylation product of metoclopramide might be neglected in comporison with that for the unchanged form.

Table I. Recovery Test of Metoclopramide and Its N^4-Conjugates in Urine

Sample No.	Substance[a] used	Added μg/ml[b]	Found μg/ml[b,c]	Recovery %
1	M	25.0	25.5	102.0
	MG	31.5	31.8	101.0
	MS	35.9	35.1	97.8
2	M	7.1	7.1	100.0
	MG	27.1	27.8	102.6
	MS	61.4	59.8	96.4
3	M	7.1	7.1	100.0
	MG	54.0	54.7	101.3
	MS	30.8	33.1	106.5

a) M, MG, and MS represent metoclopramide, metoclopramide-N^4-glucuronide, and metoclopramide-N^4-sulfonate, respectively.
b) shown as metoclopramide equivalent
c) In the case of sample No. 1, mean of 10 experments (±S.D.) for c_1, c_2, and c_3 (see Chart 1) was 25.52 ±0.50, 57.29 ±0.95, and 92.35 ±0.92, respectively.

Table II. Recovery Test of Metoclopramide and Its N^4-Conjugates in Bile

Sample No.	Substance[a] used	Added μg/ml[b]	Found μg/ml[b,c]	Recovery %
1	M	25.0	25.5	10.20
	MG	31.5	30.6	97.1
	MS	35.9	35.8	99.7
2	M	8.3	8.6	103.6
	MG	10.5	10.5	100.0
	MS	12.0	11.3	94.2
3	M	7.1	6.8	95.8
	MG	9.0	9.0	100.0
	MS	20.5	20.7	101.0

a, b) See Table I a) and b).
c) In the case of sample No. 1, mean of 10 experiments (±S.D.) for c_1, c_2, and c_3 (see Chart 1) was 25.52±0.22, 56.16±0.24, and 91.93±0.26, respectively.

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Recovery Tests in Urine and Bile

A mixture containing a known amount of every standard substance was added to the rabbit urine or vile and was separately measured by the procedure in Chart 1. The results are given in Table I and II, in which agreement between added amount and found one is reasonable. Thus, this method of separatory determination was found to be applicable for both the urine and the bile samples containing metoclopramide and its N^4-conjugates.

Quantitative Study on Metoclopramide and Its N^4-Conjugates in Rabbit Urine and Bile

The urine and bile specimens[7] collected as mentioned above were treated to determine separately metoclopramide and its N^4-glucuronide and N^4-sulfonate according to the procedure in Chart 1. The results are shown in Table III. In the urine during 6 hours after injection

Table III. Urinary and Biliary Excretion of Metoclopramide and Its N^4-Conjugates after administered intravenously 40 mg of Metoclopramide to Rabbit

Rabbit No.	Substance[a] recovered	Amount excreted in urine (mg)[b]					Amount excreted in bile (mg)[b]				
		hr[c]			Total by 6 hr		hr[c]			Total by 6 hr	
		0—1	1—3	3—6			0—1	1—3	3—6		
1 (3.5 kg)	M	1.49	1.10	1.19	3.78		0.08	0.07	0.10	0.25	
	MG	2.21	3.44	2.49	8.14	20.18	0.08	0.12	0.13	0.33	1.12
	MS	2.96	3.22	2.08	8.26		0.18	0.16	0.20	0.54	
2 (2.6 kg)	M	4.40	1.57	1.15	7.12		0.09	0.04	0.03	0.16	
	MG	2.91	2.26	1.86	7.03	19.80	0.34	0.14	0.05	0.53	1.35
	MS	2.39	1.97	1.29	5.65		0.32	0.21	0.13	0.66	
3 (2.8 kg)	M	0.61	1.22	1.69	3.52		0.07	0.04	0.05	0.16	
	MG	0.55	3.34	3.13	7.02	14.27[d]	0.45	0.39	0.25	1.09	2.10
	MS	0.38	1.75	1.60	3.73		0.34	0.24	0.27	0.85	

a) See Table I *a*).
b) shown as metoclopramide equiv.
c) time intervals of sample collection after administration
d) failed in the collection of 0—1 hr urine

of the drug solution, except for rabbit No. 3 failed in the collection of 0—1 hour urine, the total amount recovered was about 50% of the dose. Moreover, it was observed as a feature common among three rabbits that the amount of the glucuronide in the 1—6 hour urine is more predominant than the two others. In the 0—6 hour bile, on the other hand, the recovery amounted to about 3—5% of the dose is evidently small in comparison with that found in the urine. However, it is of interest that an attempt will be made to investigate the behavior of intestinal absorption for the two conjugates which account for a large part of total amount recovered.

Acknowledgement Thanks are given to Miss Shizuko Nakamura for her assistance in the experimental work.

7) That the major biliary excrements in rabbit receiving metoclopramide are the unchanged form and its N^4-glucuronide and N^4-sulfonate similar to those found in the urine was confirmed by the thin-layer chromatography.

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