[Chem. Pharm. Bull. 21(8)1747—1753(1973)]

UDC 547.495.2.09 : 615.31.033

Drug Absorption, Metabolism, and Excretion. VIII.[1] Determination of Tolbutamide and Its Metabolites in Blood and Urine

Juichiro Shibasaki, Ryoji Konishi, Toshiyuki Ueki, and Takayoshi Morishita

Faculty of Pharmaceutical Sciences, Nagasaki University[2]

(Received January 16, 1973)

The quantitative determination methods of tolbutamide (TB) and its two metabolites, namely 1-butyl-3-*p*-hydroxymethylphenylsulfonylurea (HTB) and 1-butyl-3-*p*-carboxyphenylsulfonylurea (CTB), in blood and urine were developed, which had been required especially for the detailed pharmacokinetic studies of TB involving the administration of these metabolites. These compounds were satisfactorily separated by extracting from the biological fluids with organic solvents utilizing the differences of pK_a values and distribution coefficients to organic solvents among these compounds. The quantitations were made spectrophotometrically at ultraviolet region for blood specimens and at visible region for urine specimens after the reaction with 2,4-dinitrofluorobenzene. The results of recovery tests are also given for all the procedures established.

The metabolism of tolbutamide (1-butyl-3-*p*-tolylsulfonylurea, TB), an orally active hypoglycemic agent, has been extensively studied in man and several laboratory animals. Although pronounced species differences have been recognized, the pattern of metabolic pathways in man, rabbit, guinea pig and rat appears essentially similar. In the earlier studies, 1-butyl-3-*p*-carboxyphenylsulfonylurea (CTB) has been described as an only major metabolite in these species,[3] though the production of a small amount of 1-butyl-3-*p*-hydroxymethylphenylsulfonylurea (HTB) has been pointed out.[4] More recently, however, it has become clear from the works employing ^{3}H-labeled TB[5] or ^{14}C-labeled TB[6] that HTB is a metabolite unable to be ignored quantitatively.

Several spectrophotometric methods have been reported for the determination of TB in human blood following administration of TB,[7-9] where TB was extracted from acidified blood with organic solvents prior to the quantitation without any consideration on the contamination by HTB and/or CTB, although recently, a gas-liquid chromatographic method for TB in plasma has been reported by Prescott and Redman.[10] In addition, a determination method has been reported for CTB in human urine following administration of TB,[11] in which removal of interfering TB by chloroform extraction at pH 5.5 was carried out prior to extrac-

1) Part VII: J. Shibasaki, R. Konishi, Y. Takeda, and T. Koizumi, *Chem. Pharm. Bull.* (Tokyo), **19**, 1800 (1971).
2) Location: *1-14 Bunkyo-machi, Nagasaki.*
3) L.H. Louis, S.S. Fajans, J.W. Conn, W.A. Struck, J.B. Wright, and J.L. Johnson, *J. Am. Chem. Soc.*, **78**, 5701 (1956); G. Wittenhagen and G. Mohnike, *Deut. Med. Wochschr.*, **81**, 887 (1956); T. Dorfmüller, *ibid.*, **81**, 888 (1956); G. Mohnike, G. Wittenhagen and W. Langenbeck, *Naturwissenshaften*, **45**, 13 (1958).
4) G. Wittenhagen, G. Mohnike and W. Langenbeck, *Z. Physiol. Chem.*, **316**, 157 (1959).
5) R.C. Thomas and G.J. Ikeda, *J. Med. Chem.*, **9**, 507 (1966).
6) J. Tagg, D.H. Yasuda, M. Tanabe and C. Mitoma, *Biochem. Pharmacol.*, **16**, 143 (1967).
7) *a*) H. Spingler and F. Kaiser, *Arzneim.-Forsch.*, **6**, 760 (1956); *b*) A.A. Forist, W.L. Miller, J.J. Krake and W.A. Struck, *Proc. Soc. Exptl. Biol. Med.*, **96**, 180 (1957); *c*) E. Bladh and A. Norden, *Acta Pharmacol. Toxicol.*, **14**, 188 (1958).
8) H. Spingler, *Klin. Wochschr.*, **35**, 533 (1957).
9) T. Chulski, *J. Lab. Clin. Med.*, **53**, 490 (1959).
10) L.F. Prescott and D.R. Redman, *J. Pharm. Pharmacol.*, **24**, 713 (1972).
11) E. Nelson, I. O'Reilly and T. Chulski, *Clin. Chim. Acta*, **5**, 774 (1960).

ting CTB from the strongly acidified urine. Thus, HTB has been neglected in the quantitations reported previously except the isotopic methods.[5,6] It became desirable in our laboratory to develop simple determination methods for TB, HTB and CTB in blood and urine when these are present together or alone for the purpose of investigating the pharmacokinetics of TB,[12] in which the studies following administration of HTB and CTB are involved.

The present studies deal with establishing the determination methods for 1) TB in blood when the presence of HTB and CTB is suspected as in the case of TB administration; 2) HTB and CTB in urine when they are present together as in the cases of TB and HTB administration; 3) HTB in blood when the contamination of CTB is suspected as in the case of HTB administration; 4) CTB in blood and urine without any other drug-related contaminants which are obtained after CTB administration.

Experimental

TB, HTB and CTB——TB was the gift of Japan Höchst Co., Ltd., HTB and CTB were synthesized by the following methods.

1) Synthesis of HTB[13]: This compound was prepared according to the method reported by Ohnishi[14] and followed by Tagg, *et al.*,[6] where *p*-acetoxymethylbenzenesulfonamide was reacted with *n*-butylisocyanate in dry acetone under the presence of anhydrous potassium carbonate to form 1-butyl-3-*p*-acetoxymethylphenylsulfonylurea and the latter was deacetylated in alkali to obtain HTB. The mp of HTB thus obtained was 115—118° (from MeOH–H_2O), being somewhat higher than the reported mp (101—103°,[14] 98.5—100° [6] and 110—110.5° [5]), suggesting that purification would be insufficient especially in the first two works. *Anal.* Calcd. for $C_{12}H_{18}O_4N_2S$: C, 50.40; H, 6.34; N, 9.77. Found: C, 50.45; H, 6.43; N, 9.36.

2) Synthesis of CTB[13]: CTB was prepared by Cardani, *et al.*[15] as follows: *p*-Carbethoxybenzenesulfonamide (I) was reacted with *n*-butylcarbamoyl chloride to form 1-butyl-3-*p*-carbethoxyphenylsulfonylurea (II), which was hydrolyzed in alkali to afford CTB. The present authors obtained CTB in a little different way avoiding to use *n*-butylcarbamoyl chloride. I was treated with potassium cyanate to afford 1-*p*-carbethoxyphenylsulfonylurea (III), which was condensed with *n*-butylamine to form II. Then II was hydrolyzed in alkali to afford CTB. II was also prepared by treating I with *n*-butylisocyanate.

III: The mixture of I (0.7 g), finely powdered potassium cyanate (7.0 g), and EtOH (60 ml) was refluxed for 5 hr. After removal of solvent *in vacuo*, the residue was taken up in water and a small amount of insoluble material was filtered off. The filtrate was acidified with 10% HCl and the resulting precipitate was collected by filtration. Recrystallization from aqueous methanol gave colorless plates (6.0 g, 72%), mp 167—169° (decomp.). *Anal.* Calcd. for $C_{10}H_{12}O_5N_2S$: C, 44.12; H, 4.41; N, 10.29. Found: C, 44.07; H, 4.50; N, 10.31.

II: (1) To a mixture of III (0.5 g) and *n*-butylamine (0.5 g), was added dropwise acetic acid (2.0 ml) under cooling by ice and the mixture was heated at 80° for 6 hr on a steam-bath. After cooling, water was added and the resulting precipitate was collected. Then, it was dissolved in 10% sodium carbonate and a small amount of insoluble material was filtered off. The filtrate was acidified with 10% HCl to afford a precipitate, which was recrystallized from aqueous ethanol to give colorless needles (0.3 g, 50%), mp 134—136° (decomp.) (reported mp 142—143° [15]). *Anal.* Calcd. for $C_{14}H_{20}O_5N_2S$: C, 51.22; H, 6.09; N, 8.53. Found: C, 51.27; H, 6.21; N, 8.31.

(2) To a mixture of I (0.5 g), anhydrous potassium carbonate (0.5 g), and dry toluene (8 ml), a solution of *n*-butylisocyanate (0.4 ml) in dry toluene (0.5 ml) was added dropwise at room temperature. After the addition, the mixture was heated at 110—120° for 4 hr under stirring. After cooling, water (20 ml) was added and the mixture was shaken in a separatory funnel. The aqueous layer separated was filtered to remove a small amount of insoluble substances and the filtrate was acidified with diluted HCl to afford a precipitate. Recrystallization from aqueous ethanol gave colorless needles (0.65 g, 91%), mp 132—135°, which was identical with II prepared by the method (1).

CTB: A mixture of II (5.0 g) and 7% NaOH (50 ml) was heated on a steam-bath at 80° for 1 hr. After cooling, 10% HCl was added to the mixture and the resulting precipitate was collected by suction filtration, which was recrystallized from aqueous ethanol to give colorless crystals (4.0 g, 87%), mp 211—212° (reported mp 210—211° [15]). *Anal.* Calcd. for $C_{12}H_{16}O_5N_2S$: C, 48.00; H, 5.33; N, 9.33. Found: C, 47.98; H, 5.34; N, 8.84.

12) J. Shibasaki, R. Konishi, T. Morishita and T. Ueki, *Chem. Pharm. Bull.* (Tokyo), **21**, 1754 (1973).
13) All melting points are uncorrected.
14) S. Ohnishi, *Yakugaku Kenkyu*, **34**, 107 (1962).
15) C. Cardani, E. Crescenzi, C. Pedrali and E. Adami, *Farmaco*, (*Pavia*) *Ed. Sci.*, **12**, 375 (1957), [*C. A.*, **53**, 21742^a (1959)].

Standard Drug Solutions——Each 50 mg of TB, HTB, and CTB was suspended in about 20 ml of purified water and a little excess of 0.1N NaOH was added and gently warmed. The resulting solutions were diluted to 50 ml with purified water to obtain the standard drug solutions (100 mg%).

Solvents——All solvents used were of reagent grade. Heptane and chloroform were purified by distillation. Ether was purified as follows: Ether (1 liter) was shaken with 10% sodium sulfite (100 ml) for 10 min and allowed to stand overnight. Further, the ether layer was submitted to successive washing with 10% NaOH (100 ml) and 10% HCl (100 ml) and further with water (100 ml × 5). After drying over sodium sulfate, the ether layer was distilled. Isoamyl acetate was used without purification.

0.1% 2,4-Dinitrofluorobenzene Solution (DNFB Solution)——This reagent solution was prepared by diluting DNFB of reagent grade with isoamyl acetate immediately before use.

0.5M Phosphate Buffers (pH: 5.0, 5.5, 6.0)——Buffer solutions were prepared by mixing 0.5M KH_2PO_4 solution and 0.5M Na_2HPO_4 solution.

Blank Blood and Urine——Blank blood and urine served for the preparation of standard curves were taken from male albino rabbits without giving the drugs. Blood was withdrawn from ear vein with a syringe containing 3.8% sodium citrate solution equal to one-ninth volume of blood. Urine was taken by means of bladder catheterization.

pK_a Measurement of HTB——The pK_a of HTB was determined on the basis of spectrophotometric method. Each 1.0 ml of HTB solution (2.5×10^{-4}M[16]) was added to 4.0 ml of the following media: 0.01N HCl; 0.5M phosphate buffer (pH 5.0); 0.01N NaOH. Measurements were made at 230 mμ (20°). The pK_a of HTB thus determined was 4.97.

Extractibility Measurement——The extractibility of TB, HTB, and CTB from aqueous media was tested with several organic solvents at various pH values. The solvents and pH values examined are listed in Table I. The methods of measurement are as follows.

Method 1: This method was used for all solvents except isoamyl acetate. One ml of 10 mg% drug solution was added to the mixture of 3.0 ml of purified water and 1.0 ml of either buffer solution or 3N HCl. To 4.0 ml of the solution thus obtained was added 0.2 ml of 3N HCl[17] and the optical density was measured at 230 mμ for TB and HTB, and 238 mμ for CTB using a reagent blank run through the same procedure for zero setting (OD_1). On the other hand, 5.0 ml of the samely prepared solutions as described above was shaken with 25.0 ml of each organic solvent for 20 min, and the optical density of the mixture of 4.0 ml of the aqueous layer and 0.2 ml of 3N HCl was measured as above (OD_2). The percentages of extractibility were calculated by $((OD_1-OD_2)/OD_1) \times 100$.

Method 2: This method was used for isoamyl acetate which disturbed the ultraviolet-spectrophotometric measurements as made in Method 1. To the mixture of 1.0 ml of purified water and 1.0 ml of either buffer solution or 3N HCl was added 1.0 ml of 10 mg% standard drug solution. The resulting solution was shaken with 6.0 ml of isoamyl acetate for 20 min. To 5.0 ml of the organic solvent layer was added 0.5 ml of 0.1% DNFB solution, and the mixture was heated at 145° for 7 min and cooled to room temperature. The optical density of the solution was determined at 350 mμ using a reagent blank run through the same procedure for zero setting (OD_2). On the other hand, 1.667 mg% water-saturated isoamyl acetate solution[18] was prepared for each drug, the concentration of which corresponded to that of the solution dissolving the same amount of drug as present in 1.0 ml of 10 mg% drug solution in 6.0 ml of the solvent. An 5.0 ml aliquot of the solution thus prepared was run through the procedure and the optical density was determined as above using water-saturated isoamyl acetate as a reference (OD_1). The percentages of extractibility were calculated by $(OD_2/OD_1) \times 100$.

Result and Procedure

Isolation of Each Compound from the Mixture of TB, HTB, and CTB by Organic Solvent Extraction

In order to determine each compound in the mixture of TB, HTB, and CTB, it is reasonable to isolate each compound prior to determination since the structures of these compounds are so closely related that it seems rather difficult to develop a specific method for each compound. The reported pK_a values[19] of TB and CTB are 5.32 and 3.54, respectively,

16) Prepared by diluting the standard drug solution of HTB.
17) Since the optical densities of TB and its metabolites were found to be somewhat greater in acid than in alkali, the absorbance measurements were made in the acidic medium.
18) When the solvent free of water was used, the color development was unsatisfactory.
19) A.A. Forist and T. Chulski, *Metab. Clin. Exptl.*, **5**, 807 (1956).

and that of HTB is 4.97 being determined by the present authors. Utilizing the differences of the pK_a values and the distribution coefficients between water and organic solvents of these three compounds, the isolation of each compound from the mixture was attempted to give satisfactory results. It is suggested from Table I that each compound can be practically isolated by selecting a suitable solvent and pH values. All the methods described below involve the extraction procedures based on the results presented in Table I.

Table I. Extractibility (%) of TB, HTB, and CTB from Aqueous Solutions with Various Organic Solvents at Various pH Values

Compound (pK_a)	pH[a]	Solvent				
		Ether	Chloroform	Heptane	Heptane–chloroform (8:2)	Isoamyl acetate
TB (5.43)	1.0	100	100			100
	5.0	100	98	41	89	99
	5.5	99	98	23	89	98
	6.0	98	97	11	79	98
HTB (4.97)	1.0	90	88			100
	5.0	82	74	2	5	100
	5.5	71	54	2	3	81
	6.0	46	32	1	1	69
CTB (3.54)	1.0	100	85			100
	5.0	43	6	0	1	52
	5.5	10	2	0	0	12
	6.0	1	0	0	0	0

a) The solution of pH 1.0 was prepared by adding 3N HCl and those of pH 5.0—6.0 by 0.5M phosphate buffers.

Reexamination of Determination Methods of TB

The present authors reexamined the two methods reported for TB determination in order to apply not only to TB but to HTB and CTB. The one is based on the ultraviolet absorption of TB (UV method)[7] and the other is based on the color produced by the reaction of TB with 2,4-dinitrofluorobenzene (DNFB method).[8]

(1) **UV Method**——This method has been first reported by Spingler and Kaiser[7a] and succeedingly by the other investigators.[7b,c] In all the methods so far reported, the organic solvents used for the TB extraction from blood were evaporated to dryness and the extracts were again dissolved in ethanol or methanol for spectrophotometric measurements. The authors simplified the procedure by reextracting the organic solvent layer with alkaline or buffer solution followed by measuring the absorbance of the acidified aqueous layer. The maximum wave lengths were seen at 230 mμ for both TB and HTB, and at 238 mμ for CTB where the estimations were carried out. Since the absorbance due to normal blood constituents was found to be low and constant, but high and unfixed with blank urine even when it was diluted to 20 times, this method seemed to be applicable to blood samples but not suitable for urine samples.

(2) **DNFB Method**——This method has been first described by Spingler[8] for the determination of TB in serum and later applied to the determination of CTB in urine by Nelson, *et al.*[11] In these studies, TB and CTB were extracted from the acidified biological fluids with isoamyl acetate, then the organic solvent layer was added with 0.1% DNFB solution and the resulting mixture was heated at 150° for 5 min followed by measuring the absorbance at 380 mμ. Isoamyl acetate has been served not only as an excellent solvent for the extraction of TB and CTB but also as a reaction solvent for the color development. The urine blank due to this method was as low as the reagent blank when urine was diluted over 20 times.

This method, therefore, is applicable to the determination of TB and its metabolites in urine. Some modifications described below were made on the previous methods.

1) It was found that the reagent blank could be lowered by reducing the volume of DNFB solution used by the previous workers by half keeping the linearity of standard curves of TB and its metabolites, 2) The effects of reaction temperature and time on color development were also reexamined and the reaction at 145° for 7 min was proved to be preferable to that at 150° for 5 min, the conditions employed by the previous workers, 3) Since it was confirmed that the absorption spectra of the colored products with TB and its two metabolites had a maximum at 350 mμ and the corresponding absorbances were much greater than those of a plateau at about 380 mμ, the authors used the wave length of 350 mμ for the measurements, while the previous workers used that of 380 mμ.[8,11]

Determination of TB in Blood Contaminated with HTB and CTB

Since the pK_a values of TB and HTB are comparatively close and considerably greater than that of CTB, the extraction of the mixture of these three compounds with ether or chloroform at pH above about 5.5 is assumed to be an useful method for dividing both TB and HTB from CTB as indicated by Table I. Ether or chloroform, however, extracts TB and HTB together. For isolating TB from HTB, the selection of extraction solvent would be a clue. It is suggested from Table I that heptane and heptane–chloroform (8: 2) are able to extract TB selectively at pH 5.0 leaving almost all HTB in aqueous layer, the latter being preferable to the former because of its higher extracting ability for TB. Though the amount of TB extracted decreased by the addition of blood, the linearity of standard curve was kept as shown in Fig. 1.

Procedure——One ml of citrated blood specimen (containing 0.1 ml of 3.8% sodium citrate solution) was hemolyzed with 3.0 ml of purified water and added with 1.0 ml of pH 5.0 phosphate buffer in a 50 ml glass-stoppered centrifuge tube. The mixture was shaken with 25.0 ml of heptane–chloroform (8: 2) for 20 min. After centrifugation, 20.0 ml of the organic solvent phase was shaken with 5.0 ml of 0.1N NaOH for 20 min and then centrifuged. Four ml of the alkaline layer was added with 0.2 ml of 3N HCl and the absorbance was measured at 230 mμ using a blood blank for the zero setting. The blood blank according to this method gave an absorbance of about 0.05 against water. For the preparation of standard curve, 1.0 ml of citrated blank blood was mixed with 1.0 ml of standard TB solution (2.0, 4.0, 6.0, 8.0, and 10.0 mg%), 2.0 ml of purified water, and 1.0 ml of pH 5.0 phosphate buffer and then the mixture was treated as above. The standard curve thus obtained is shown in Fig. 1 (curve b).

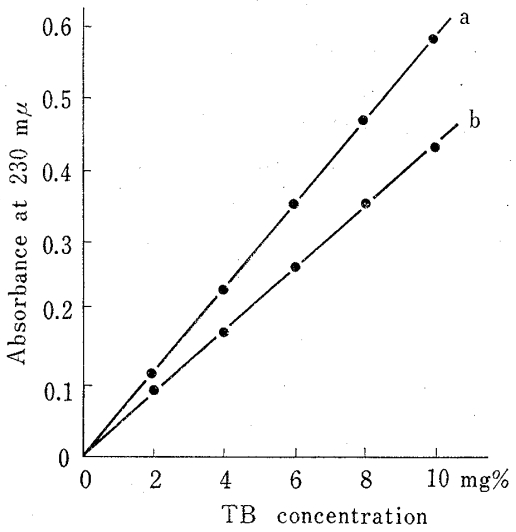

Fig. 1. Standard Curve of TB in Blood
a : TB without blood
b : TB with blood

Determination of HTB in Blood Contaminated with CTB

It was indicated from Table I that shaking a mixture of HTB and CTB with ether or chloroform at pH 6.0 served to extract HTB selectively but with unsatisfactory recovery and although the amount of HTB extracted was improved by extracting at pH 5.0, the contamination of CTB could not be neglected. But fortunately, this contamination was proved negligible as demonstrated in Fig. 2 when the ether extraction at pH 5.0 was made in the presence of blood. This phenomenon may be interpreted in terms of the affinity of CTB to serum or blood cells under these conditions, but the details remain unknown.

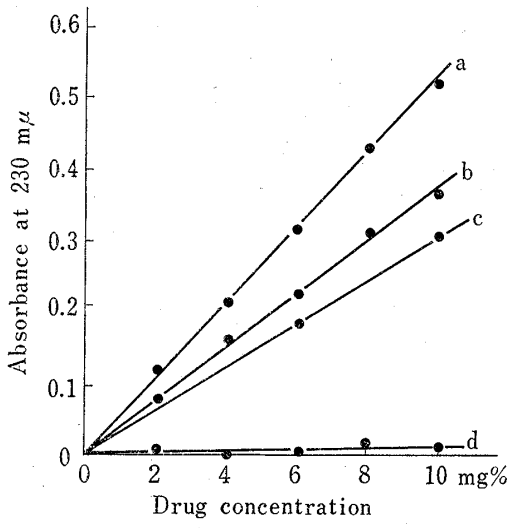

Fig. 2. Standard Curve of HTB in Blood

a : HTB without blood c : CTB without blood
b : HTB with blood d : CTB with blood

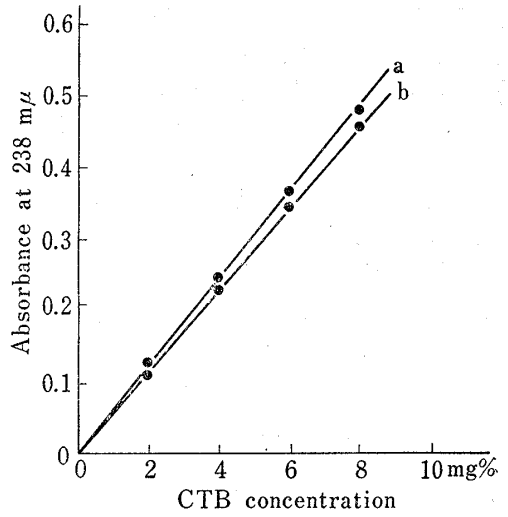

Fig. 3. Standard Curve of CTB in Blood

a : CTB without blood
b : CTB with blood

Procedure——The procedure is quitely same as that for TB in the preceeding section except using ether as the extraction solvent. The blood blank according to this method gave an absorbance of about 0.09 against water. The standard curve for HTB in blood is shown in curve b of Fig. 2.

Determination of CTB in Blood without Other Contaminants

As indicated from Table I, CTB was satisfactorily extracted only from a strongly acidic solution. Ether extraction of CTB from blood under the strong acidification, however, resulted in the reddish-brown coloration of the organic solvent layer. If the ether layer was shaken with NaOH solution to return CTB into aqueous layer, the color was also transferred together with CTB and interfered the estimation of CTB at the ultraviolet region. On the contrary, the color remained in the organic solvent layer when 0.5M phosphate buffer (pH 5.5) was employed as an aqueous medium to return CTB. Although the amount of CTB extracted by ether decreased slightly by the addition of blood, the standard curve obeyed Beer's law as shown in Fig. 3.

Procedure——One ml of citrated blood specimen (containing 0.1 ml of 3.8% sodium citrate solution) was hemolyzed with 3.0 ml of purified water and added with 1.0 ml of 3N HCl in a 50 ml glass-stoppered centrifuge tube. The mixture was shaken with 25.0 ml of ether for 20 min. After centrifugation, 20.0 ml of the organic solvent phase was shaken with 5.0 ml of 0.5M phosphate buffer (pH 5.5) for 20 min and then centrifuged. Four ml of the buffer layer was added with 0.2 ml of 3N HCl and the absorbance was measured at 238 mμ using a blood blank for zero setting. The blood blank according to this method gave an absorbance of about 0.09 against water. The standard curve was prepared by the same method described above for TB and shown in curve b of Fig. 3.

Each Determination of HTB and CTB in Urine Containing Both the Compounds

HTB can be extracted selectively from an aqueous mixture of HTB and CTB by shaking with isoamyl acetate at pH 6.0 as indicated from Table I. Since the procedure isolating CTB alone from the mixture would become too complicated to use as a routine analytical method, simultaneous extraction of HTB and CTB was carried out from strongly acidified solution and the sum of absorbance due to both the compounds was measured from which the concentration of CTB was calculated as described below. It was found that the amounts of HTB and CTB extracted were not affected by the presence of blank urine when it was diluted

over 20 times. The results of the recovery experiments where known amounts of HTB and CTB were added to blank urine are shown in Table II. Here, the recovery seems to be almost satisfactory. Method 2 described below is also applicable to the determination of CTB in urine without contamination of other drug-related compounds.

Table II. Recovery of Added HTB and CTB from Aqueous Solutions

Added (μg)		Recovered (μg)	
HTB	CTB	HTB	CTB
10	90	11.3	91.7
10	90	11.5	87.8
10	90	11.7	87.9
20	80	20.9	78.2
20	80	21.4	82.1
30	70	31.4	68.4
30	70	32.6	68.0
50	50	50.7	52.7
50	50	50.9	53.5
50	50	52.0	47.5
50	50	48.0	52.6
70	30	72.0	27.1
70	30	70.0	32.9
80	20	79.3	22.8
90	10	91.0	10.3
90	10	91.1	12.2

Procedure——(1) Determination of HTB (Method 1): To 2.0 ml of urine specimen diluted over 20 times was added 1.0 ml of 0.5M phosphate buffer (pH 6.0) in a 25 ml glass-stoppered test tube, and then the mixture was shaken with 6.0 ml of isoamyl acetate for 20 min. Five ml of the organic solvent layer was reacted with 0.5 ml of 0.1% DNFB solution at 145±1° for 7 min in another test tube and allowed to stand at room temperature for 1 hr. The absorbance was measured at 350 mμ using a urine blank for zero setting. The urine blank according to this method gave an absorbance of about 0.20 against water which was essentially same as that of a reagent blank. For the preparation of standard curve, 1.0 ml of the standard HTB solution (2.0, 4.0, 6.0, 8.0, and 10.0 mg%) was mixed with 1.0 ml of purified water and 1.0 ml of 0.5M phosphate buffer (pH 6.0) and the mixture was run through the above procedure.

(2) Determination of the Sum of HTB and CTB (Method 2): To 2.0 ml of urine specimen diluted over 20 times was added 1.0 ml of 3N HCl in a 25 ml glass-stoppered test tube, and the mixture was run as in method 1. The urine blank was also same as obtained by method 1. The standard curves of HTB and CTB were prepared by the same method described above in method 1.

(3) Calculation of the Concentration of CTB: The concentration (mg%) of HTB is E_1/f_1 where E_1 is the absorbance of urine specimen and f_1 is that corresponding to 1.0mg% HTB standard solution by method 1. The concentration of CTB (mg%) is calculated from $(E_2-f_2E_1/f_1)/f_3$ where E_2 is the absorbance of urine specimen by method 2 and f_2 and f_3 are the absorbances corresponding to 1.0 mg% HTB and CTB standard solutions by method 2, respectively. It would be recommended to determine the values of f_1, f_2, and f_3 at each deter mination. These values were practically calculated from those of 10.0 mg% standard drug solutions and the typical values for f_1, f_2, and f_3 were 0.036, 0.060, and 0.060, respectively.

Acknowledgement The authors wish to thank Japan Höchst Co., Ltd. for a gift of tolbutamide and K. Kitaoka and A. Kugiyama for their technical assistances.