Chem. Pharm. Bull. 16(9)1726—1731(1968)

UDC 615.212.033:034:615.31

Drug Absorption, Metabolism, and Excretion. II.[1] Metabolism of Bucetin (β-Hydroxybutyro-*p*-phenetidide) in Rabbits

Juichiro Shibasaki, Tamotsu Koizumi, Terukazu Tanaka, and Michiko Nakatomi

Faculty of Pharmaceutical Sciences, Nagasaki University[2]

(Received December 26, 1967)

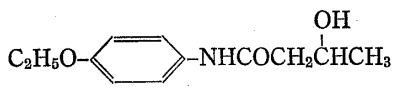
Metabolism of an antipyretic and analgesic of aniline derivative, bucetin (β-hydroxybutyro-*p*-phenetidide, I*a*) in rabbits was studied.

From the results of estimations of total glucuronic acid and sulfate in the urine, it was deduced that the majority of metabolites was excreted as glucuronides and the excretion of sufates was negligiblly small, if any.

By treating the urine with β-glucuronidase, extracting with ether, and purifying the extract through silica gel column, N-(β-hydroxybutyro)-*p*-aminophenol (Ib) and *p*-hydroxyacetanilide (IIIb) were isolated and identified with authentic samples. Moreover, *p*-hydroxyacetoacetanilide (IIb) was identified in the ether extract by thin-layer chromatography.

For the formation of IIIb, there were two reactions involved; de-ethylation of Ia at ethoxyl group and the loss of two carbons in β-hydroxybutyryl group. Since the latter reaction was not familiar for drug metabolism, the mechanism of the reaction was also discussed in this communication.

Bucetin (β-hydroxybutyro-*p*-phenetidide, Ia),[3] an aniline derivative containing β-hydroxybutyryl group instead of acetyl group in phenacetin has been noted as an excellent antipyretic and analgesic with low toxicity and now being used therapeutically.



Ia

In the first paper of this series,[1] the kinetics of metabolism and excretion of acetanilide and *p*-hydroxyacetanilide was reported. In order to perform the kinetic studies on this new drug like other analgesics of aniline derivatives mentioned above, it is necessary to know the detail of the metabolic pathways of the drug, of which, however, few information is available to date except the work in rabbits by Ichibagase, *et al.*[4]

Therefore, the study on the metabolism of Ia in rabbits has been attempted to provide satisfactory results, which are to be reported in this communication.

Quantitative Investigation of the Metabolites in the Urine

Ia (400 mg/kg) was suspended in water and administered to rabbits orally through the catheter inserted to the stomach. The urine was collected for a period of 24 hours and estimations were made for total glucuronic acid and sulfate in the urine. The results are shown in Table I.

Compared with the normal urine, the increase of total glucuronic acid was remarkable, which clearly showed that the considerable amount of glucuronide was excreted in the urine.

1) Part I: J. Shibasaki, T. Koizumi, and T. Tanaka, *Chem. Pharm. Bull.* (Tokyo),**16**, 1661 (1968).
2) Location: *4–23 Bunkyo-cho, Nagasaki.*
3) *a*) G. Ehrhart and H. Ott, U.S. Patent 2830087 (1958) [*C.A.*, **52**, 14662*g* (1958)]; *b*) G. Doll and E. Hackenthal, *Arzneim. Forsch.*, **13**, 68 (1963) [*C.A.*, **59**, 3234*f* (1963)]; *c*) G. Ehrhart, E. Lindner, and A. Haeussler, *ibid.*, **15**, 727 (1965) [*C.A.*, **63**, 13879*h* (1965)].
4) H. Ichibagase, S. Kojima, and S. Tsuzuki, Meeting of Kyushu Branch, Pharmaceutical Society of Japan, Fukuoka, July 1967.

On the other hand, the increase of sulfate was small and therefore, the sulfate in the metabolites was considered negligible, if any.

On determining the total amount of conjugated *p*–hydroxyacetanilide in the urine after the dosis of acetanilide, Brodie, *et al.*[5] acidified the urine with hydrochloric acid and heated in an autoclave for 1.5 hours at 15 pounds pressure. The resulting *p*–aminophenol was estimated by an indophenol method. When the procedure was applied to the Ia urines, approximately one half of the dose was recovered as *p*–aminophenol as shown in Table I.

In order to avoid the loss due to imperfect absorption in the case of oral administration, 100 mg of Ia dissolved in water was injected intravenously to rabbits. Urine collection was made for a period of 10 hours and *p*–aminophenol was estimated following the procedure described above. Almost all of the amount administered was recovered as shown in Table I. These facts clearly suggest that the ethoxyl group of Ia is perfectly de–ethylated to form hydroxyl group. Further, the urine hydrolyzed as above was extracted with ether after being adjusted to pH 7.0 and it was confirmed that thin–layer chromatography (TLC) of the ether extract revealed only one spot corresponding to *p*–aminophenol, as shown in Fig. 1, Eh.

Table I. Glucuronide, Ethereal Sulfate, and *p*–Aminophenol Output in Rabbits receiving β–Hydroxybutyro–*p*–phenetidide (Ia) and N–(β–Hydroxybutyro)–*p*–aminophenol (Ib)

Dose	% of dose excreted after administration[a]		
	Glucuronide[b]	Etherael sulfate[b]	*p*–Aminophenol[c]
Ia 400 mg/kg orally	62(46—81)[d]	2(0—4)[e]	51[f]
Ia 100 mg/body intravenously	—[h]	—[h]	98(87—111)[g]
Ib 400 mg/kg orally	60[f]	5[f]	—[h]

a) 24 hr urine after oral administration and 10 hr urine after intravenous administration were estimated.
b) The values given are calculated from the differences between the amounts for 24 hr urine after dosing of Ia or Ib and the normal amounts for 24 hr taken as the means for 48 hr before dosing.
c) The values given are the total amounts of metabolites which are hydrolyzed to *p*–aminophenol by heating the acidified urine at 15 pounds pressure for 1.5 hr.
d, e, g) The values given are the average for 3, 2, and 5 rabbites respectively, the ranges being in parentheses.
f) estimation for one rabbit
h) The estimation was not carried out.

Identification of the Metabolites in the Urine

TLC of the ether extract of the urine collected during 24 hours after the administration of Ia (400 mg/kg) to rabbits gave only one spot (spot ***c***) in Fig. 1, E, which was also detected in the urine treated with β–glucuronidase and will be discussed later.

Then, in order to investigate the glucuronides which were considered the major metabolites from the results of glucuronic acid estimation, the following experiments were carried out. Aqueous suspension of 2100 mg of Ia was administered to two rabbits (400 mg/kg) and the urine was collected for a period of 24 hours. Ninety percent of the excreted urine was adjusted to pH 5.0 with acetic acid and 10.0 ml of β–glucuronidase solutiom (13000 units/ml)[6] was added to it. The mixture was incubated for 18 hours at 38° and subjected to continuous extraction with ether for 30 hours after saturating with ammonium sulfate. The ether layer was dried over sodium sulfate and evaporated under a reduced pressure to give crystalline residue (about 0.5 g), which revealed three spots by TLC (***a***, ***b***, and ***c*** of Fig. 1, E_g). This residue was dissolved in a small portion of acetone, mixed with 1.0 g of silica gel and the

5) B.B. Brodie and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **94**, 22 (1948).
6) Tokyo–Zoki Chemical.

mixture after removal of the solvent in vacuum was charged on the top of a column of silica gel (10. 0 g). The column was successively eluted with 100 ml of benzene, 100 ml of benzene–ethyl acetate (8:2), 100 ml of benzene–ethyl acetate (6:4), and 500 ml of benzene–ethyl acetate (5:5). Fractions of 10 to 20 ml each were collected and examined by TLC.

Almost nothing was obtained by the first three solvents, but three metabolites corresponding to ***a***, ***b***, and ***c*** of Fig. 1, E_g were eluted by benzene–ethyl acetate (5:5) with the respective order. Although the amount of ***a*** was too samll to be isolated, ***b*** and ***c*** were obtained as crystalline forms. These metabolites were identified as follows.[7]

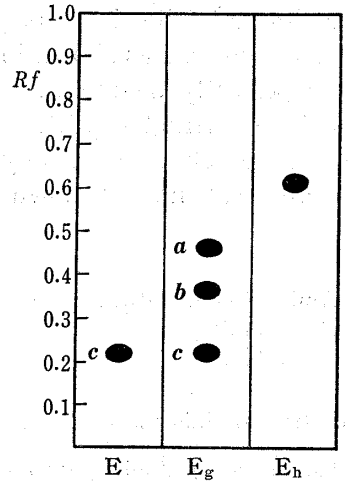


Fig. 1. Thin–Layer Chromatograms of β–Hydroxybutyro–*p*–phenetidide (Ia) Urine

E: ether extract of untreated urine
E_g: ether extract of urine subjected to hydrolysis with β–glucuronidase
E_h: ether extract of urine treated with HCl at 15 pounds pressure for 1.5 hr
a: *p*–hydroxyacetoacetanilide (IIb)
b: *p*–hydroxyacetanilinde (IIIb)
c: N–(β–hydroxybutyro)–*p*–aminophenol (Ib)
the spot in E_h: *p*–aminophenol
solvent system: benzene–ethyl acetate (3:7)
spraying agent: 5% aqueous silver nitrate

a: *Rf* value and coloring in TLC coincided with those of the authentic *p*–hydroxyacetoacetanilide (IIb).[8]

b: The fractions exhibiting this spot in TLC were collected to give 30 mg of a raw material. Recrystallization from ethyl acetate–benzene gave light yellowish needles (mp 168—170°), which was identified with *p*–hydroxyacetanilide (IIIb) by mixed melting point and comparisons of infrared (IR) spectrum and *Rf* value of TLC. Besides, the results of elementary analysis of this metabolite accorded with IIIb. *Anal.* Calcd. for $C_8H_9O_2N$: C, 63.57; H, 5.91; N, 9.27. Found: C, 62.89; H, 5.91; N, 9.37.

c: The fractions exhibiting this spot in TLC were collected to give about 80 mg of raw substance. Recrystallization from ethyl acetate–petroleum ether gave colorless cubes (mp 87—90°). After drying on phosphorus pentoxide under reduced pressure at 60° for 48 hours, it became to show mp 114—116°. Both undried and dried substances gave the identical *Rf* value of TLC and IR spectrum. The substance of lower melting point appears to contain water of crystallization and that of higher melting point to be anhydride. These characteristics relative to melting point, IR spectrum, and *Rf* value in TLC coincided with those of the authentic N–(β–hydroxybutyro)–*p*–aminophenol (Ib).[8] Analytical results of the metabolite after drying were as follows, which accorded with those of anhydrous Ib. *Anal.* Calcd. for $C_{10}H_{13}O_3N$: C, 61.54; H, 6.67; N, 7.18. Found: C, 61.12; H, 6.69; N, 7.23. Spot ***c*** of Fig. 1, E, which was obtained from ether extract of untreated urine corresponded to this metabolite.

From the results recorded above, it is deduced that the major metabolites of Ia are the glucuronides of Ib and IIIb and that there also exists small amount of glucuronide of IIb.

7) Existence of unchanged Ia, acetoacetyl–*p*–phenetidide (IIa),[8] phenacetin (IIIa), and *p*–aminophenol was examined by TLC because the metabolic pathways were assumed as will be mentioned, but these substances were not found (the coloration of Ia and IIIa are, however, not so sensitive that they might have been overlooked when the quantities were not so large).

8) Preparation of the authentic samples is to be published in part III of this series of study.

That the glucuronide of IIIb was perfectly hydrolyzed to *p*–aminophenol by heating in the acidified medium under 15 pounds pressure for 1.5 hours, is beyond doubt from the work of Brodie, *et al.*[5] and study of the present authors.[1] Glucuronides of Ib and IIb are reasonably considered to be hydrolyzed to *p*–aminophenol under the same condition.[9] Therefore, this deduction concerning about the metabolic pathways is able to explain consistently the experimental fact that 100% of the drug was recovered as *p*–aminophenol from the hydrolyzed urine which was collected during 10 hours after intravenous administration of 100 mg of Ia.

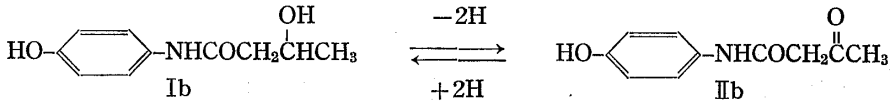
Metabolism of Ib

Metabolism of Ib in rabbits, which has been proved to be the major metabolite of Ia, was investigated. Aqueous solution of Ib (400 mg/kg) was given to rabbits through oral route and the examination were carried out on the urine excreted during 24 hours after administration. The amount of glucuronic acid and sulfate excreted were estimated with the same procedure described above for Ia, to afford almost the same results as the case of Ia, as shown in Table I. TLC of the ether extracts of untreated and *β*–glucuronidase treated urine equally gave the distinct spot of unchanged Ib. Moreover, the spots of IIb and IIIb were observed on the TLC of ether extract of *β*–glucuronidase treated urine. From these results it was confirmed that the identical metabolites were excreted in the urine after the administration of Ia and Ib.

Discussion of the Metabolic Pathways of Ia

(i) Formation of Ib: Formation of Ib by de–ethylation of ethoxyl group of Ia and subsequent glucuronic acid conjugation of Ib are most reasonably predictable route of metabolism.

(ii) Formation of IIb: Generally speaking, the fact is well known that aliphatic secondary alcohols reversibly convert to the corresponding ketones in the living body. Therefore, the existence of the following reaction is reasonably presumed.



(iii) Formation of IIIb: In the formation of IIIb from Ia, there are two reactions involved; de–ethylation of Ia at ethoxyl group and the loss of two carbons of *β*–hydroxybutyryl group. As almost identical metabolites were obtained after the administration of Ia and Ib, existence of the route (Ia→Ib→IIIb) is clear where de–ethylation takes place first. As the metabolic route from IIIa to IIIb has already been observed in rabbits,[10] the other metabolic route *via* IIIa (Ia→IIIa→IIIb) may not be neglected in which the removal of carbon atoms takes place first, but experimental proof for the latter route has not been made yet.

The loss of two carbons in the side chain is not a familiar reaction for drug metabolism. As the similar reaction de–alkylation of O–alkyl or N–alkyl group through *α*–hydroxylated intermediate is recalled. These de–alkylations are, however, the results of cleavage of C–O or C–N bond and not the cleavage of C–C bond like the present case.

Hydrolysis of anilides (Ia or Ib) to corresponding anilines followed by acetylation is appeared to be a possible mechanism for this reaction.

Furthermore, the following pathways (Chart I) are presumed for another reasonable explanation, since each of the steps presented is well–known biochemical reaction.

At first, the secondary alcohol (Ia or Ib) is converted to the corresponding ketone (IIa or IIb) as described above. *ω*–Oxidation of the ketone produces *α*–ketonic acid, which through decarboxylation and oxidation[11] results in *β*–ketonic acid and finally decarboxylation

9) Although the hydrolysis was not tested directly with glucuronides of Ib and IIb, the complete conversion of Ib and IIb to *p*–aminophenol under the same condition was confirmed by means of TLC.

10) J.N. Smith and R.T. Williams, *Biochem. J.*, **44**, 239 (1949).

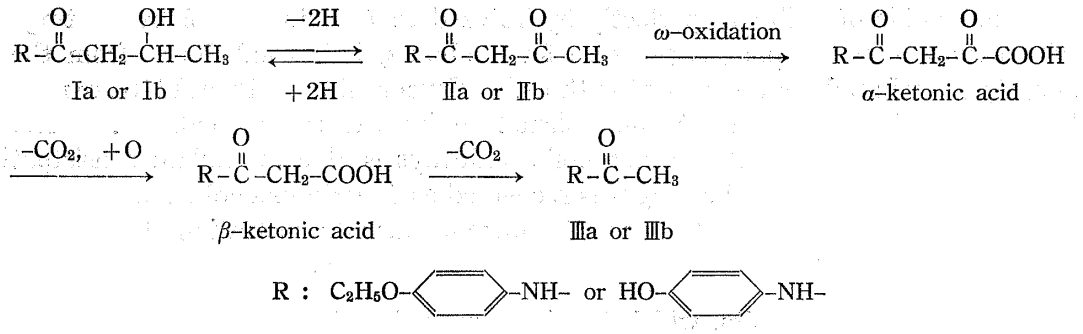


Chart 1

of the β-ketonic acid[12] leads to the production of IIIa or IIIb. If IIIa is formed, it is obvious that IIIa is de-ethylated to IIIb.

Studies are in progress to clarify the mechanism of the removal of the carbon atoms in the side chain.

Overall metabolic pathways described above are summarized and shown in Chart 2.

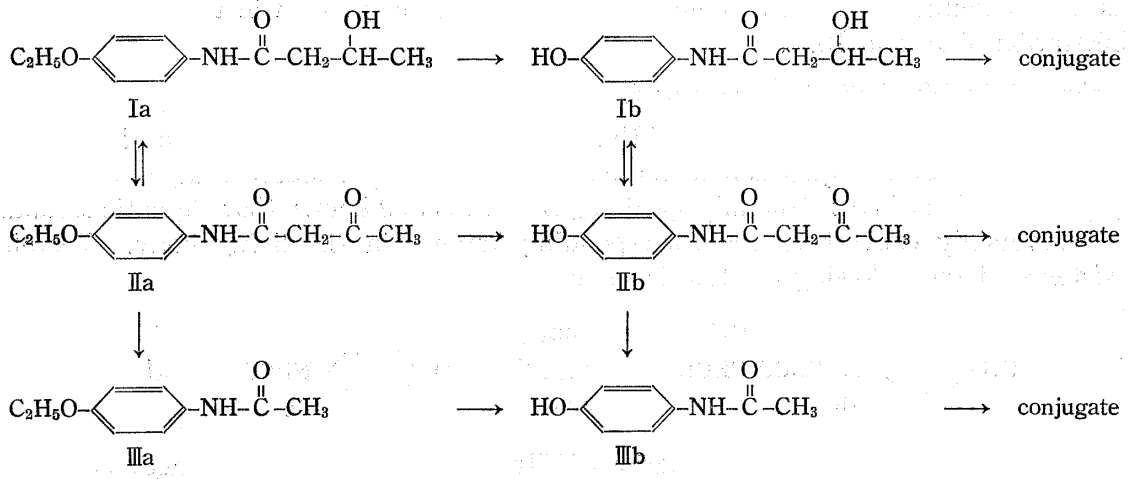


Chart 2

Considerations on the Pharmacological Activity of Ia

From the fact that Ia is partially metabolized to IIIb, it may be deduced that the anti pyretic and analgesic action of Ia is somewhat due to this metabolite, as was deduced by Brodie, *et al.* with acetanilide[13] and phenacetin.[14] On the other hand, according to the results by Conney, *et al.*[15] that the antipyretic and analgesic activity of IIIa is not due to its metabolite IIIb, but to IIIa itself, the possibility must be taken into account that the pharmacological action of Ia comes from IIIa, which is probably the intermediate in the metabolic pathways from Ia to IIIb. In addition, for discussing on the activity of Ia, the activities of Ia itself and of Ib, the major metabolite of Ia, cannot be neglected, but informations of them are not available to date.

11) For example, the last step of; threonine $\xrightarrow{-H_2O}$ imino acid $\underset{-NH_3}{\overset{+H_2O}{\rightleftharpoons}}$ α-ketobutyric acid $\underset{+O}{\overset{-CO_2}{\longrightarrow}}$ propionic acid.

12) For example, the second step of; β-hydroxybutyric acid $\underset{+2H}{\overset{-2H}{\rightleftharpoons}}$ acetoacetic acid $\xrightarrow{-CO_2}$ acetone.

13) B.B. Brodie and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **94**, 29 (1948).

14) B.B. Brodie and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **97**, 58 (1949).

15) A.H. Conney, M. Sansur, F. Soroko, R. Koster, and J.J. Burns, *J. Pharmacol. Exptl. Therap.*, **151**, 133 (1966).

Therefore, further investigation is necessary to clarify the whole aspect of the mechanism of the pharmacological activity of Ia.

Experimental

Procedure——Male rabbits maintained on "okara" and weighing 2.5 to 3.5 kg were used in the experiments. In case of oral administration of Ia, 400 mg/kg of the drug was ground finely with mortar and pestle, suspended in about 20 ml of water and given to rabbits through the catheter inserted to stomach. In case of Ib, same amount of the drug dissolved in about 20 ml of water was given similarly. In case of intravenous administration of Ia, 100 mg of the drug was dissolved in about 80 ml of water and injected intravenously through the ear vein of rabbits. Naturally excreted urine was collected in the bottle containing a few drops of xylene. At the desired time the urine remained in the bladder was collected by using Nelaton's catheter and combined with the urine naturally excreted by that time.

Authentic Samples——Bucetin (β-hydroxybutyro-*p*-phenetidide), mp 157—158° (ref.[3a] mp 160°) was the gift of Takeda Chemical Industries, Ltd. Phenacetin and *p*-hydroxyacetanilide were purchased. The other samples were synthesized by the authors.[8]

Quantitative Analysis of Total Glucuronic Acid in the Urine——Naphthoresorcinol method reported by Mead, *et al.*[16] was employed.

Quantitative Analysis of Sulfate in the Urine——Gravimetric method reported by Otaki[17] was employed with a slight modification using Gooch's crucible instead of analytical filter paper for separating barium sulfate.

Quantitatiev Analysis of *p*-Aminophenol after Hydrolysis of Metabolites in the Urine——Followed the method reported by Brodie, *et al.*[5] outline of which was described in the previous section of this report.

Thin-Layer Chromatography (TLC)——Silica gel B-5 of Wako Pure Chemical Industries, Ltd. was spread about 250 μ thick and dried at 110° for 1.5 hours. *Rf* values and coloration of the authentic samples are summarized and shown in Table II.

Table II. *Rf* Values and Color Reactions of Authentic Samples in Thin-Layer Chromatography

Compound	*Rf* value in benzene–ethyl acetate		Color reaction	
	(5:5)	(3:7)	$AgNO_3$[a]	$FeCl_3$[b]
β-Hydroxybutyro-*p*-phenetidide (Ia)	0.23	0.38	none	yellowish brown
N-(β-Hydroxybutyro)-*p*-aminophenol (Ib)	0.12	0.22	black	brown
Acetoacetyl-*p*-phenetidide (IIa)	0.54	0.64	brown	gray
p-Hydroxyacetoacetanilide (IIb)	0.25	0.46	black	gray
Phenacetin (IIIa)	0.42	0.59	none	yellowish brown
p-Hydroxyacetanilide (IIIb)	0.22	0.37	black	dark brown
p-Aminophenol	0.45	0.61	dark brown	purple

a) Detected by spraying 5% aqueous silver nitrate.
b) Detected by spraying 10% aqueous ferric chloride followed by heating at 120° for 5 min.

Acknowledgement The authors are grateful to Mrs. H. Mazume for elementary analysis and Mr. H. Ishikawa for IR measurement. This work was partly supported by the Grant-in-Aid for Scientific Research from the Ministry of Education, to which the authors are grateful. The authors wish to express their deep gratitude to Takeda Chemical Industries, Ltd. for the kind supply of bucetin.

16) J.A.R. Mead and R.T. Williams, *Biochem. J.*, **69**, 61 (1958).
17) T. Otaki, *Yakugaku Zasshi*, **74**, 1107 (1954).