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BIOCHEMICAL EFFECTS OF AROMATIC COMPOUNDS—III

FERRIHAEMOGLOBINAEMIA AND THE PRESENCE OF *p*-HYDROXY-*o*-TOLUIDINE IN HUMAN BLOOD AFTER THE ADMINISTRATION OF PRILOCAINE

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Abstract—The kinetics of the formation of ferrihaemoglobinaema and hydroxylated derivatives of prilocaine were studied in man after the subcutaneous administration of the drug. There was a close time relationship between the amount of ferrihaemoglobin formed and the concentration of the *p*-hydroxytoluidine in plasma. This metabolite was recovered in urine as 34 per cent of the prilocaine dose; corresponding amounts of *o*-toluidine and the *o*-hydroxytoluidine were 0·75 and 2·7 per cent, respectively. Nitroso or hydroxylamino metabolites were not detected.

DURING work with a local anaesthetic agent prilocaine (α-propylamino-2-methylpropionanilide) it was found that some of the patients became cyanotic without any evidence of deficient oxygen supply or blood circulation. Measurements have given evidence for a dose dependent ferrihaemoglobinaemia induced by the drug.[1,2] However, prilocaine added to human erythrocytes *in vitro* does not induce ferrihaemoglobin production.[1] Thus it can be assumed that metabolites of the compound are responsible for the effect *in vivo*. It has been found that prilocaine among other things is metabolized to *o*-toluidine and its *orto*- and *para*-hydroxylated metabolites in animals,[3] and it is known that these hydroxylated metabolites induce ferrihaemoglobin *in vitro* and *in vivo* in animals and in man.[4,5]

It has been the aim of the present investigation (a) to establish the existence of *o*-toluidine and its hydroxylated derivatives in blood and urine from healthy individuals given prilocaine and (b) to study the kinetics of the formation of such possible metabolites and the concomitant ferrihaemoglobinaemia.

MATERIALS AND METHODS

Volunteers. Five healthy medical students, four men and one woman, 24–28 years old were given 20 mg of prilocaine per kg of body weight by subcutaneous infiltration into the thigh. The men had blood haemoglobin values between 12·7–14·7 g/100 ml and the woman 12·5 g/100 ml. They were fasted and immobilized during the first 3 hr of the experiment and about 400 ml of sterile physiological saline was administered intravenously during the same time to ensure sufficient urine volumes. After 4 hr they

were allowed to move freely. They had meals mainly consisting of yoghurt and sandwiches after about 4, 9 and 13 hr. Blood and urine samples were taken before the administration of the drug. Blood samples were then taken at intervals over the following 24 hr and urine was collected quantitatively in 8–10 portions during the same period.

Blood was taken in heparinized tubes and immediately chilled in an ice bath. The samples were centrifuged at 800 *g*, 0° during 10 min, after 30–60 min of storage.

Plasma was used for the determination of metabolites of prilocaine, erythrocytes for the determination of ferrihaemoglobin.

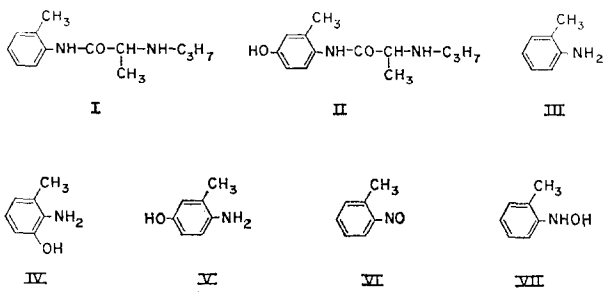

FIG. 1. The chemical structure of prilocaine (I) and some of its derivatives, *p*-Hydroxyprilocaine (II), *o*-Toluidine (III), *o*-Hydroxytoluidine (IV), *p*-Hydroxytoluidine (V), 2-Nitrosotoluene (VI), 2-Hydroxylaminotoluene (VII).

Urine was stored in polystyrene bottles at +4°, the fresh urine was immediately transferred to these bottles. Bottles containing full portions of urine were succesively frozen and accordingly stored at −20°, until analysis. The urine samples were analysed for the presence of metabolites of prilocaine. Determination of methaemoglobin was done using the method of Evelyn & Malloy.[6]

Reference substance for chromatographic procedures. The following reference substances were used and are referred to below by the appropriate roman numerals, cf also Fig. 1.

I. Prilocaine, α-propylamino-2-methylpropionanilide × HCl
II. *p*-Hydroxyprilocaine, α-propylamino-2-methyl-4-hydroxypropionanilide × HCl
III. *o*-Toluidine
IV. *o*-Hydroxytoluidine, 2-amino-3-hydroxytoluene
V. *p*-Hydroxytoluidine, 2-amino-5-hydroxytoluene
VI. 2-Nitrosotoluene
VII. 2-Hydroxylaminotoluene.

The compounds were synthetized at AB ASTRA, Södertälje, Sweden, or purchased (V) from Fluka AG, Buchs SG. Before use, they were recrystallized and chromatographed in methanol–benzene acetic acid (8:45:4). Their melting points were also taken.

Chromatographic identification of metabolites of prilocaine in urine. For the search of unconjugated metabolites of prilocaine 5 ml of alkalinized (pH = 8) urine was extracted in 50 ml ether–benzene (1:1). The organic phase was removed after shaking

and then evaporated to a suitable volume, 0·03 ml. This volume, after adding a small amount of ethanol, was used for thin layer chromatography on silica or aluminium oxide. The upper phases of the following mixtures were used as solvents: 45 vol. of benzene, 4 vol. of glacial acetic acid and 8 vol. of methanol in the case of the Silica gel and 66 vol. of acetone, 5 vol. of ammonia and 32 vol. of ligroine in the case of the aluminium oxide gel. The metabolites were located by spraying with a solution of the sodium salt of naphtoquinone sulphonic acid. During about 60 min (Silica gel) and 30 min (aluminium oxide gel) of chromatography at +25° the solvent front ascended about 18 cm.

In order to free the metabolites from possible conjugates, another portion of the urine was acidified (6 ml urine + 1 ml N HCl) and heated for 4 hr at 120° (controls showed that this treatment did not affect prilocaine or its metabolites). The hydrolysed urine was thereafter extracted and chromatographed as described above.

Quantitative analysis of prilocaine and metabolites of prilocaine in plasma and urine. The analyses were done on hydrolysed as well as on non-hydrolysed biological samples to get some information of the relative amounts of conjugated and unconjugated metabolites.

o-*Toluidine* (*III*). This was assayed as described for aniline by Brodie and Axelrod.[7] It was extracted from the biological sample into benzene, returned to acid, diazotized and coupled with *N*-(1-naphtyl)ethylene diamine. The resulting dye was acidified and the absorbancy read at 660 nm. *o*-Toluidine, added to urine or plasma in amounts of 2–100 μg, was recovered to 94 per cent, range 89–99 per cent, n = 5. The minimal detectable amount was 0·6 μg/ml plasma or urine. The simultaneous presence of the *orto*- and *para*-hydroxylated derivatives of *o*-toluidine did not interfere with the dye reaction.

o-*Hydroxytoluidine* (*IV*). This was determined as previously described by Ragnarsson and Wistrand.* It was first stabilized by acetylation of the amino group, the acetylated product was extracted in ether, then alkalinized and treated with 2,6-dichloroquinone chloroimide. The resulting blue colour was measured at 620 nm. The recovery after adding 600 μg to urine was 70 per cent, range 65–75 per cent, n = 5. The minimal detectable amount was 0·6 μg/ml plasma or urine. The presence of equimolar amounts of *o*-toluidine and its *p*-hydroxy derivative gave no interference.

Occasionally a blue colour also developed in the control urine and plasma with an optical density corresponding to that seen for about 0·5 μg/ml of *o*-hydroxytoluidine. No effort was made to identify this interfering substance.

p-*Hydroxytoluidine* (*V*). This was determined according to the method described for *p*-aminophenol by Brodie and Axelrod.[7] It was extracted into ether, then returned to acid and coupled with phenol in the presence of sodium carbonate according to Otani, Akagi and Sakamoto[8] to form an indophenol dye which was assayed at 635 nm. Recoveries of *p*-hydroxytoluidine added to plasma and urine amounted to 76 per cent, range 71–81 per cent, n = 5, if the amount added was above 10 μg; the recovery was 50 per cent on the average if the added amount was 2–5 μg. Hydrolysis was done after the addition of the compound. The minimal detectable amount was 0·5 μg/ml plasma or urine. The presence of *o*-toluidine and *o*-hydroxytoluidine interfered with the reaction only if their concentrations were 2 times higher than that of *p*-hydroxytoluidine.

* B. Ragnarsson and P. Wistrand, in preparation.

RESULTS

The plasma concentration of p-*hydroxytoluidine and the ferrihaemoglobinaemia after the administration of prilocaine.* The plasma concentration of *p*-hydroxytoluidine and the ferrihaemoglobinaemia observed in the subjects after the administration of prilocaine is shown in the upper parts of Fig. 2A–E. Rather similar curves were obtained in all five subjects both with respect to the time course and the magnitude of the changes of the *p*-hydroxytoluidine concentration and the ferrihaemoglobinaemia that appeared. The ferrihaemoglobinaemia paralleled that of the *p*-hydroxytoluidine with the peak value of the ferrihaemoglobinaemia 1 hr after that of the

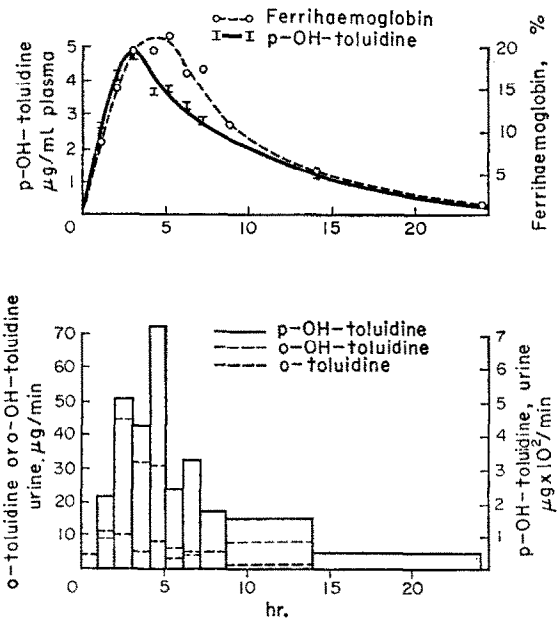

FIG. 2A

prilocaine metabolite. Phase plane plots for the *p*-hydroxytoluidine concentration and the concomitant ferrihaemoglobinaemia revealed slightly different regression lines at increasing and decreasing concentrations of *p*-hydroxytoluidine (Fig. 4).

o-Toluidine or *o*-hydroxytoluidine were not detected in plasma with the method used.

Chromatographic identification of prilocaine metabolites in the urine. Typical chromatograms are shown in Fig. 3A and B. The control urine samples did not show spots corresponding to the reference substances. Such spots, however, were observed in urine samples taken between 2 and 5 hr after the subcutaneous administration of prilocaine. In the non-hydrolysed urine prilocaine (I), *p*-hydroxyprilocaine (II) and *o*-toluidine (III) were easily identified on Silica gel (Fig. 3A). No such good resolution of the prilocaine (I) and *o*-toluidine (III) spots was achieved on aluminium oxide gel (Fig. 3B).

Hydrolysis of the urine caused the appearance of additional spots corresponding to those of *p*- and *o*-hydroxytoluidine (IV, V). No spots corresponding to nitroso- or

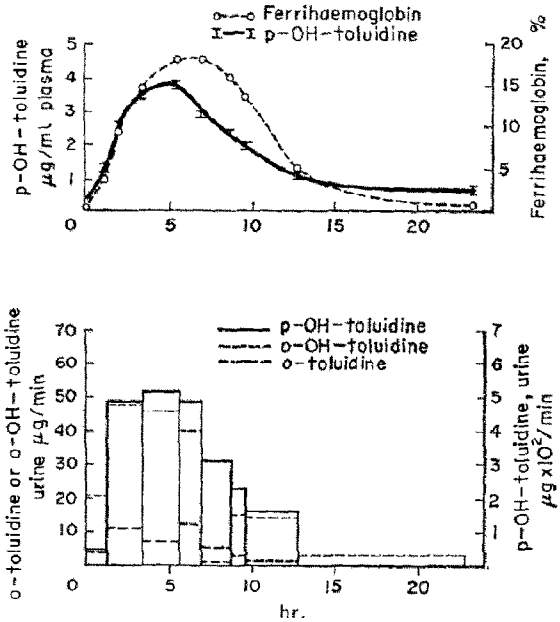
Ferrihaemoglobin
p-OH-toluidine
p-OH-toluidine µg/ml plasma
Ferrihaemoglobin, %
p-OH-toluidine
o-OH-toluidine
o-toluidine
o-toluidine or o-OH-toluidine urine µg/min
p-OH-toluidine, urine µg x10²/min
hr.

FIG. 2B

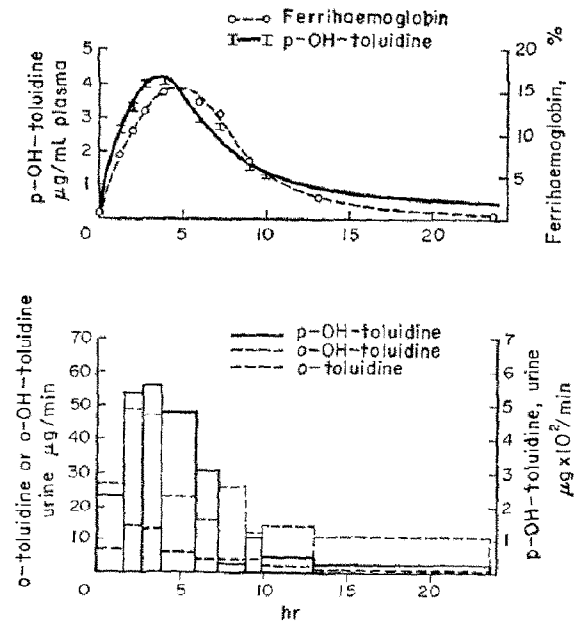
Ferrihaemoglobin
p-OH-toluidine
p-OH-toluidine µg/ml plasma
Ferrihaemoglobin, %
p-OH-toluidine
o-OH-toluidine
o-toluidine
o-toluidine or o-OH-toluidine urine µg/min
p-OH-toluidine, urine µg x10²/min
hr

FIG. 2C

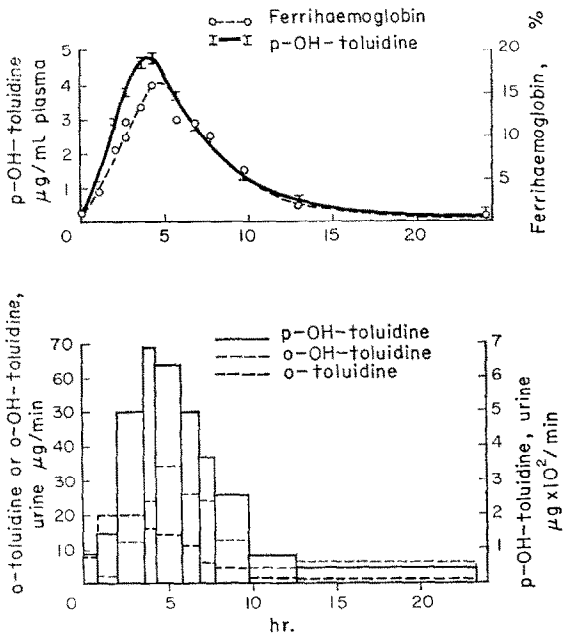

FIG. 2D

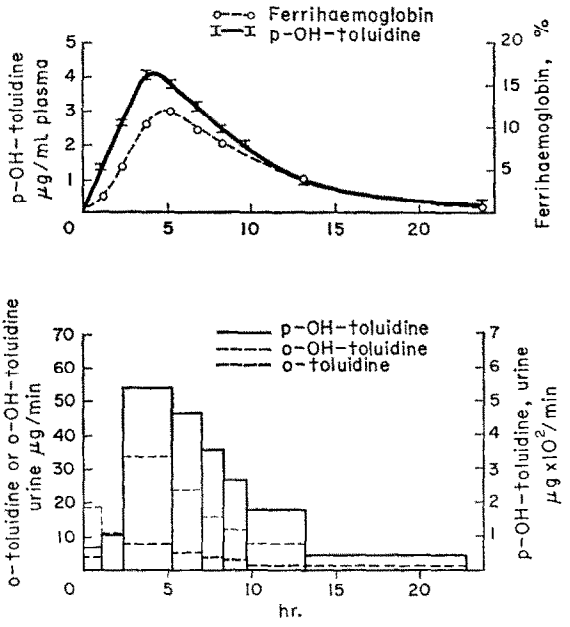

FIG. 2E

FIG. 2. The appearance of ferrihaemoglobinaemia and prilocaine metabolites in plasma and urine after the administration of 20 mg per kilogram body weight of prilocaine to five healthy adults. The ferrihaemoglobinaemia was calculated as per cent of the total concentration of haemoglobin in blood.

hydroxylaminotoluene (VI, VII) were found in the hydrolysed or non-hydrolysed urine. The R_f-values and their variations for the authentic substances run on several occasions, are seen in Table 1. These values should be compared with the R_f-values of the corresponding spots of the urines.

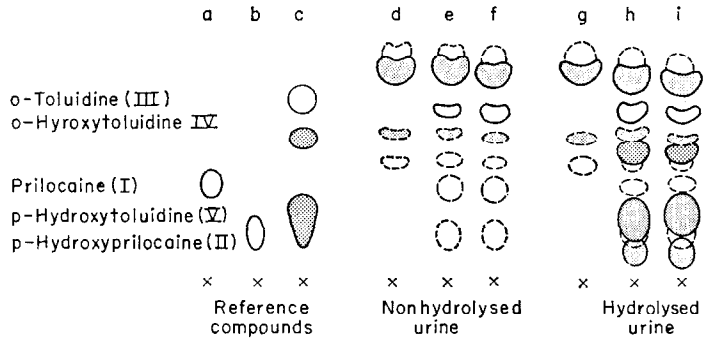

FIG. 3A

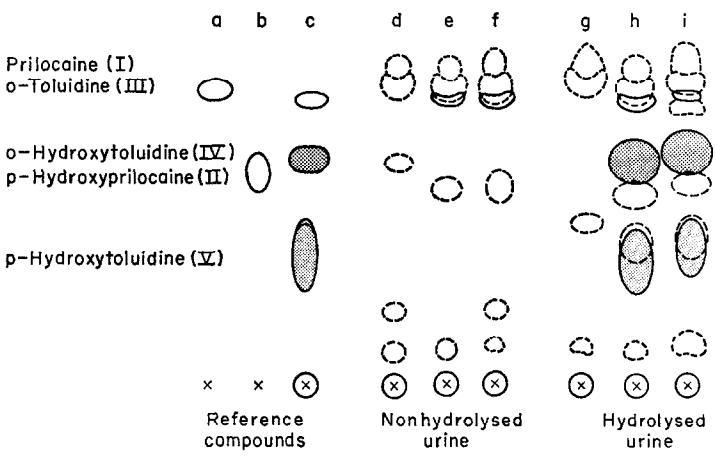

FIG. 3B

FIG. 3. Chromatograms of reference compounds and of urines from two subjects (Nr A and B, of Figs. 2 and 4) on Silica gel (Fig. 3A) and aluminium gel (Fig. 3B). (a) Prilocaine (I), (b) *p*-Hydroxyprilocaine (II), (c) Mixture of *o*-toluidine (III), *o*-hydroxytoluidine (IV), *p*-hydroxytoluidine (V), (d) Nonhydrolysed urine from subject B before the administration of prilocaine, (e) Nonhydrolysed urine from subject A collected during the first 2 hr after the administration of prilocaine, (f) Nonhydrolysed urine from subject B collected during 2 and 3·5 hr after the administration of prilocaine, (g) Hydrolysed urine from subject B before the administration of prilocaine, (h) Hydrolysed urine from subject A collected during the first 2 hr after the administration of prilocaine, (i) Hydrolysed urine from subject B collected during 2 and 3·5 hr after the administration of prilocaine.

Quantitative determination of the urinary excretion of prilocaine metabolites. The presence of the above metabolites in the urine (Table 2) was also secured by the colorimetric techniques, which moreover allowed a quantitation of the excretion at least of the conjugated metabolites. *p*-Hydroxytoluidine (V) was excreted in the largest amounts

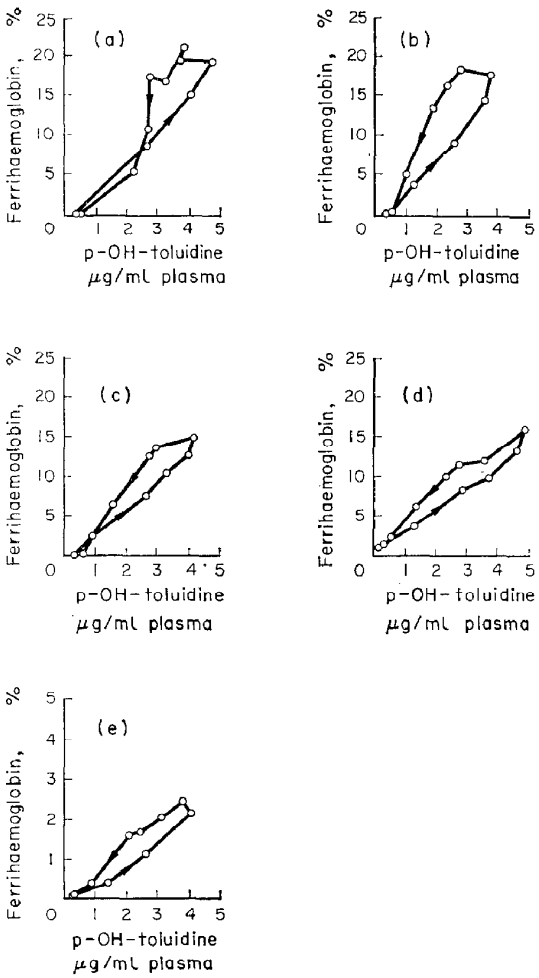

FIG. 4. Phase plane plots of the plasma concentration of *p*-hydroxytoluidine and the corresponding ferrihaemoglobinaemia after the administration of prilocaine. The arrows indicate the time sequence of the values from time zero.

(255 mg) and about 35 per cent (mean of all patients) of the injected prilocaine was calculated as being excreted as this metabolite. Only a minor fraction appeared in the urine as the unconjugated substance. *o*-Hydroxytoluidine (IV) appeared in much (about 13 times) smaller amounts than the *p*-hydroxyderivative (V). Some *o*-toluidine (III) was also detected in the non-hydrolysed urine. Half the excreted amounts of the hydroxylated metabolites occurred in the urine on an average at 7·4 hr after the injection (range 6–11 hr); no obvious difference was seen between the *o*- and *p*-hydroxymetabolites (IV, V). The excretion of *o*-toluidine (III) was more rapid, half the amount being excreted at about 3 hr after the prilocaine (I) injection. The excretion curves are seen in the lower parts of Fig. 2A–E.

TABLE 1. R_f-VALUES FOR PRILOCAINE AND ITS METABOLITES AND FOR SPOTS FROM NONHYDROLYSED AND HYDROLYSED URINE FROM HEALTHY SUBJECTS BEFORE AND AFTER THE ADMINISTRATION OF 20 mg PER kg BODY WEIGHT OF PRILOCAINE (I)

Reference compound	R_f-values*, Silica gel			R_f values*, Aluminium oxide gel		
		Spots in urine			Spots in urine	
	Reference compound	Non-hydrolysed	Hydro-lysed	Reference compound	Non-hydrolysed	Hydro-lysed
Prilocaine (I)	27 (28–29)	28 (27–29)	28 (27–30)	85 (84–89)	?†	?†
p-Hydroxyprilocaine (II)	15 (13–16)	14 (14–15)	14†	62 (59–65)	59 (56–64)	?†
o-Toluidine (III)	53 (52–54)	52 (50–54)	52 (50–54)	84 (82–88)	83 (82–85)	83 (81–85)
o-Hydroxytoluidine (IV)	40 (39–41)	Absent	38 (38–39)	40 (38–44)	Absent	42 (37–46)
p-Hydroxytoluidine (V)	18 (15–19)	Absent	18 (17–19)	68 (66–71)	Absent	67 (64–70)

* The mean value and the range for five subjects and the corresponding reference compounds.
† The actual spots were not easily delimited from other spots of nonprilocaine origin in the urine.

TABLE 2. THE AMOUNT OF PRILOCAINE METABOLITES EXCRETED IN URINE DURING A 24 HR PERIOD AFTER THE ADMINISTRATION OF 20 mg PER kg BODY WEIGHT OF PRILOCAINE (I) TO HEALTHY SUBJECTS

Subjects A–E	Excreted amount in mg			
	o-Toluidine (III), total mount	*o*-Hydroxy-toluidine (IV), total amount	*p*-Hydroxy-toluidine (V), total amount	*p*-Hydroxy-toluidine (V), unconjugated
Mean value	5·02	15·6	254·8	0·19
Prilocaine dose (%)	0·75	2·7	34·2	0·03

DISCUSSION

Aromatic compounds can principally be metabolized *in vivo* to metabolites hydroxylated at the ring structure or by the oxidation of the amino group to a nitroso group. Both hydroxylated and nitroso compounds are known to induce ferrihaemoglobinaemia *in vitro* and *in vivo*[4,5,8] by a catalytical mechanism.

The finding that the aromatic compound prilocaine induced ferrihaemoglobinaemia in man[1,2] initiated studies on the *in vivo* metabolism of the compound, since it was found that the drug itself was inert in this respect.[1] These investigations[3] were initially performed in different animals and it was found[3,6] that many but not all animals metabolized prilocaine to *o*-toluidine (III) and its *o*- and *p*-hydroxyderivatives (IV, V). There was a close correlation between the ability to produce *p*-hydroxytoluidine (V) and the magnitude of the ferrihaemoglobinaemia *in vivo*. In the present

investigation similar studies have been performed in man. It is apparent that prilocaine is converted to hydroxylated derivatives of *o*-toluidine also in this species, and that *p*-hydroxytoluidine (IV) appears to be the main metabolite, about 30 per cent of a dose of prilocaine being converted to that compound and excreted in the urine mainly in the conjugated form. There is a close correlation between the plasma concentration of *p*-hydroxytoluidine (V) and the concomitant ferrihaemoglobinaemia with slightly different slopes for the regression lines at increasing and decreasing plasma concentrations of the prilocaine metabolite. The explanation for this could be a relative inertness of the ferrihaemoglobin reducing system i.e. the ferrihaemoglobin reductase system does not respond immediately when the concentration of the ferrihaemoglobin-inducing metabolite decreases.

The fact that *p*-hydroxytoluidine (V) induces ferrihaemoglobinaemia in human erythrocytes *in vitro* does not necessarily mean that this metabolite is the ferrihaemoglobin inducing compound *in vivo* since, (a) conjugated hydroxylated metabolites do not induce ferrihaemoglobinaemia *in vitro*; (b) the amount of nonconjugated *p*-hydroxytoluidine (V) excreted in the urine is low and (c), thus the concentration of the unconjugated form of the compound in plasma seems to be low also.

However, *in vitro* experiments* with human erythrocytes have shown that steady state concentrations of *p*-hydroxytoluidine (V) in the range of 0·01–0·03 mg/ml will suffice to induce ferrihaemoglobinaemia of about the same magnitude and with the same rate as in the subjects of the present study. Thus it seems likely that a low plasma concentration of nonconjugated *p*-hydroxytoluidine (V) being a function of the total plasma concentration of the compound measured, is mainly responsible for the ferrihaemoglobinaemia induced by prilocaine.

Nitroso or hydroxylamino metabolites (VI, VII) of prilocaine were not detected in blood or urine from the subjects, in accordance with previous animal studies. Even after large (50 mg/kg) intravenous doses of *o*-toluidine to cats, such metabolites could not be recovered in plasma using a polarographic method. It cannot be excluded, however, that there were also low but efficient plasma concentrations of these metabolites (VI, VII) *in vivo*, not detectable with the methods used and that these metabolites contributed to induce the ferrihaemoglobinaemia. *In vitro* experiments have shown that nitrosotoluidine (VI) induces a ferrihaemoglobin production in intact erythrocytes at a very low concentration, 20 μg/ml, which is far below the limit of detection by the methods used in the present study.

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