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METABOLISM OF KETOTIFEN BY HUMAN LIVER MICROSOMES

In Vitro Characterization of a Tertiary Amine Glucuronidation

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ABSTRACT:

Biotransformation of ketotifen was investigated *in vitro* using human liver microsomes. Three of the four metabolic pathways observed *in vivo* in man were exhibited under the conditions of incubation, namely demethylation, *N*-oxidation, and *N*-glucuronidation, the absent route being the ketoreduction, which probably has a cytosolic localization. The kinetic parameters of the *N*-glucuronidation (K_M for ketotifen and UDPGA and V_{max}) were determined with native and detergent-treated microsomes. Treatment by Triton X-100 increased by about 3-fold the conjugation reaction. No sex difference was observed and *N*-glucuronidation did not seem to be inhibited either by bilirubin or by 4-nitrophenol. Thus, human liver microsomes are a useful and suitable *in vitro* model for studying metabolic routes, specific for man, as in the case of ketotifen. Obviously, the results obtained can only reflect partially the multiplicity of *in vivo* events and interpretation has to be complemented by investigations with other models.

UDP-glucuronyltransferase (EC 2.4.1.17) is an enzyme involved in metabolic pathways for both endogenous (bilirubin, steroids) and exogenous substrates. UDP-glucuronyltransferase catalyzes the conjugation of UDP-glucuronic acid to numerous functional groups, the most significant being alcohols, phenols, carboxylic acids, thiols, and amines. Biotransformation studies *in vivo* with cyproheptadine (1), tripelennamine (2), cyclobenzaprine (3), a methyl pyridine-substituted dioxane (LY 108380) (4), and ketotifen (5) revealed a novel route of drug metabolism: the *N*-glucuronidation of tertiary amines giving rise to quaternary ammonium compounds (6). In these studies, *N*-quaternary glucuronides were found only in urine of humans and higher primates (7). Other species (rat, dog, rhesus monkey) did not excrete this new type of glucuronide in appreciable amounts.

In rats, two UDP-glucuronyltransferases have been characterized: one of them was active on substrates like 4-nitrophenol and was inducible by 3-methylcholanthrene, the other conjugating bilirubin and induced by phenobarbital (8, 9). This classification is roughly in agreement with the one proposed by Wishart *et al.* (10), separating activities in "late-foetal" and "neonatal" groups according to their ontogenesis. In man, liver microsomes have been shown to conjugate numerous substrates *in vitro*, with velocities comparable to those observed in laboratory animals (11). Nevertheless, the small number of studies reported did not allow those authors to show whether UDP-glucuronyltransferase activities were supported by one, two, or more enzymes.

In this report, the metabolism of ketotifen (Zaditen), a new type of antianaphylactic agent, and particularly the kinetics of its *N*-glucuronidation was investigated *in vitro* using human liver microsomes.

Materials and Methods

Materials. [^{14}C]Ketotifen, specific acitity, 107 μCi/mg, purity >98%, labeled at the C-4 position of the benzocycloheptathiophen ring, was supplied by Sandoz Ltd. (Basel, Switzerland). Spherisorb 10 ODS came from Phase Sep (New York), UDPGA[1] (sodium salt), β-glucuronidase (bovine liver) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). NADP, G-6-P, G-6-PDH came from Boehringer (Mannheim, West Germany).

Preparation of Microsomal Fractions. Human livers were obtained from kidney transplantation donors. Livers were quickly removed after circulatory arrest, frozen in dry ice, and further stored at −80°C. Microsomes were prepared from thawed liver as previously described; under these experimental conditions, enzymatic activities have been shown previously to be well preserved (12).

Incubation Procedure. Except when otherwise stated, incubations were performed in 1 ml of 50 mM sodium phosphate buffer, pH 7.4, with 5 mM $MgCl_2$, containing 100 nmol [^{14}C]ketotifen, 5 μmol UDPGA, and about 2 mg of microsomal protein. After 30 min, the reaction was stopped by addition of 2 ml of acetonitrile and the tubes were vigorously shaken. Proteins were pelleted by a 10-min centrifugation at 5000*g*. Under these conditions, pellets retained no radioactivity. Supernatants were counted for total radioactivity, evaporated to dryness under nitrogen, and used for analysis of metabolites by HPLC (see below). Blanks were performed by omitting either microsomes or UDPGA. For kinetic constant determinations, the concentration of ketotifen and UDPGA varied from 2 to 250 μM and from 100 to 5000 μM, respectively.

The effect of protein concentration on the formation rate of metabolites was investigated with concentrations varying from 0.5 to 6 mg and the time dependence by varying incubation times between 10 and 60 min. All determinations were performed in duplicate with native microsomes and with microsomes treated by 1 volume of 0.3% Triton X-100 for 30 min at +4°C (the final concentration of Triton in the incubation medium was 0.06%).

Monooxygenase activity toward ketotifen was tested by adding a NADPH-generating system consisting of 0.15 mM NADP, 2.5 mM G-6-P, 1 U/ml G-6-PDH in the presence or in the absence of UDPGA to the incubation medium. Blanks were then performed by omitting the NADPH-generating system. Inhibition studies were performed by adding either 0.04 mM bilirubin or 0.1 mM 4-nitrophenol, both dissolved in 0.015 M sodium hydroxide to the incubations containing 1.5 mg protein of Triton-treated microsomes. Control incubations were performed by addi-

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[1] Abbreviations used are: UDPGA, UDP-glucuronic acid; UDPGT, UDP-glucuronyltransferase; G-6-P, glucose-6-phosphate; G-6-PDH, glucose-6-phosphate dehydrogenase.

tion of an equal volume of 0.015 M sodium hydroxide. It has been verified that the presence of sodium hydroxide did not alter the rate of formation of the *N*-quaternary glucuronide.

Analytical Procedure. Unchanged drug and metabolites were analyzed by reversed phase HPLC. The HPLC system consisted of two Altex 110 A pumps controlled by an Altex 421 programmer (Beckman France) and a Berthold LB 503 continuous radioactivity detector (Wildbad, West Germany). An analytical 300 × 4.6 mm i.d. column packed with Spherisorb 10 ODS (particle size, 10 μm) was used. Elutions were performed with a programmed multistep gradient of 0.01 M ammonium carbamate solution and methanol. The proportion of methanol to ammonium carbamate solution was 10% (v/v) at first and increased to 20, 47.5, 70, and 100% after 27, 42, 51, and 60 min, respectively. The total time for elution was 70 min, including the final 10 min with 100% methanol. The solvent flow rate was kept constant at 2 ml/min. Dry samples were dissolved in 300 μl of H_2O-methanol, 50:50 (v/v) mixture. After filtration, 200 μl were injected. The radioactivity of the column effluent was detected by use of a glass scintillator cell. The efficiency of the radioactivity detector was 25% and the limit of detection was 1000 dpm which correspond to 4.2 ng (10 pmol) [^{14}C]ketotifen.

Retention times of the formed metabolites were compared with those of reference samples. Polar metabolites were analyzed before and after incubation with β-glucuronidase. Metabolites were collected and their structures confirmed by mass spectrometry as described elsewhere (13). Briefly, a Nermag R 10-10B Sidar 11A gas chromatography-mass spectrometer supplemented by a PDP/8 computer system (Nermag, Rueil-Malmaison, France) was used. The chromatograph was equipped with a glass solid injector and a glass capillary column (30 m × 0.3 mm i.d.) coated with SE 52 (Chrompack, Middleburg, The Netherlands). The spectra were taken in the electron ionization mode. The ionization voltage was 70 eV and the emission current was 250 μA. Parent drug and metabolites were analyzed before or after incubation with β-glucuronidase.

TABLE 1

Metabolism of ketotifen

In vitro incubations were conducted for 30 min as described in *Materials and Methods* with 3 mg microsomal proteins and 0.07 mM [^{14}C]ketotifen. Results are expressed as pmol metabolites formed in 1 min by 1 mg protein for one typical experiment.

NADPH Generating System	5 mM UDPGA	*N*-Quaternary Glucuronide	*N*-Oxide	Nor-ketotifen
Native microsomes				
+	−	<1	38	251
−	+	41	<1	<1
+	+	16	39	282
Triton-treated microsomes				
+	−	<1	3	110
−	+	154	2	<1
+	+	48	6	105

Results

Metabolic Pathways. The different metabolites of ketotifen formed *in vitro* after incubation with human liver microsomes are displayed in table 1 and fig. 1.

In the presence of a NADPH-generating system, microsomes catalyzed the formation of *N*-oxidized ketotifen and mainly of nor-ketotifen. Microsomes catalyzed the formation of *N*-glucuroketotifen when UDPGA was added in the absence or presence of a NADPH-generating system. In the latter instance, the formation of the glucuronide was reduced, suggesting a competition between oxidation and conjugation reactions. When incubations were carried out with ketotifen and cofactors in the absence of microsomes, no metabolites were formed.

Nor-ketotifen had the longest retention time in HPLC (74 min) and was characterized by GLC/MS by the molecular ion at m/z = 295 ($M\cdot^+$) and a characteristic ion fragment at m/z = 253.

N-oxidized ketotifen was a minor metabolite. Its retention time was 51 min and it decomposed itself during GLC/MS analysis giving unchanged drug. *N*-Glucuroketotifen formed *in vitro* had the same HPLC retention time (22 min) as the glucuronide found in the urine of treated patients. The structure of this *N*-quaternary glucuronide formed *in vivo* has been previously proved by different methods, including NMR spectroscopy, which showed that the glucuronic acid residue was coupled to the nitrogen atom of the piperilydene ring forming a *N*-quaternary derivative (5). Glucuronides obtained from both *in vivo* and *in vitro* experiments formed

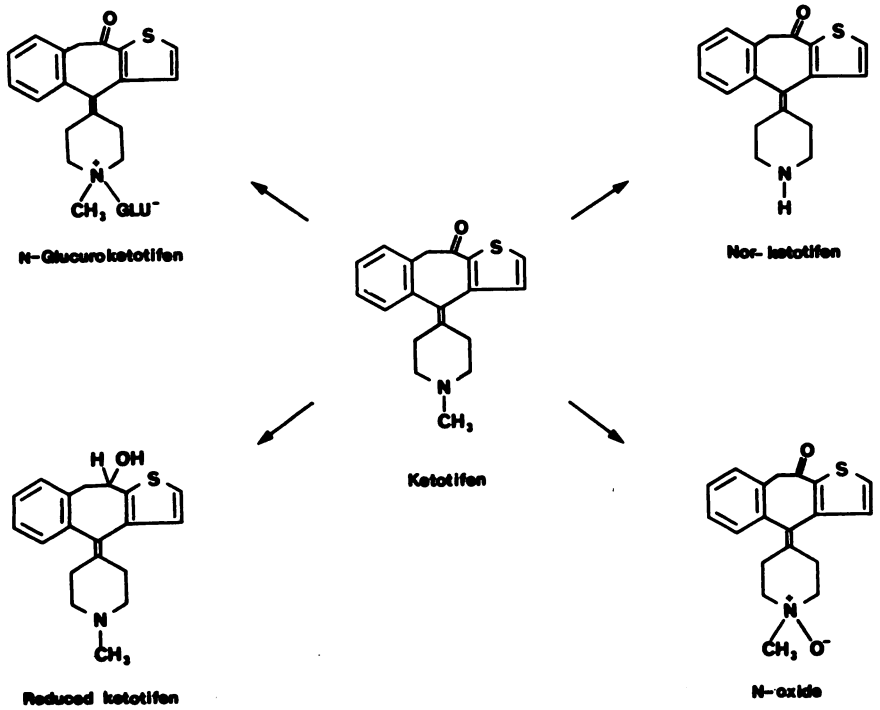

FIG. 1. *Proposed major metabolic pathways of ketotifen in man*

unchanged ketotifen after incubation with β-glucuronidase (HPLC retention time = 61 min ($M\cdot^+$), m/z = 309 in GLC/MS).

Triton X-100, a nonionic detergent, exerted a dual action on *in vitro* metabolism. It inhibited the formation of N-oxide and norketotifen and strongly enhanced the *N*-glucuronidation. However, in the presence of Triton X-100, a competition between oxidation and conjugation pathways was observed again.

To further characterize the conjugation reaction, its kinetic parameters were determined.

Time Dependence and Effect of Protein Concentration on the *N*-Glucuronidation. As shown in fig. 2, the reaction was linear up to 60 min with both native and detergent-treated microsomes, and up to 6 and 1.5 mg protein, respectively, with native and detergent-treated microsomes. This latter result was verified with microsomal preparation from a different liver. The rate of formation of the *N*-quaternary glucuronide was a function of the protein concentration during incubation, even if the velocity (expressed as pmol of glucuronide formed in 1 min by 1 mg protein) was different. Thus, all further determinations were performed for 30 min with 1.7 to 2.6 mg protein for native microsomes and with 1.2 to 1.8 mg for detergent-treated microsomes.

Kinetic Parameters of the *N*-Glucuronidation. Results of these determinations are displayed in table 2 and fig. 3. With native microsomes, two K_M for ketotifen were observed at 12.5 and 100 μM. After treatment with detergent, UDP-glucuronyltransferase exhibited only one K_M value at about 40 μM. Simultaneously, the V_{max} of detergent-treated microsomes was greatly enhanced compared to the two V_{max} of untreated microsomes. By varying the UDPGA concentrations from 0.1 to 5 mM, only K_M and one V_{max} were obtained with both native and detergent-treated microsomes (table 2). At lower concentrations of UDPGA, the glucuronidation rate was too low to be accurately determined. As expected, treatment with detergent increased the V_{max} but also the K_M from 250 to 440 μM. The V_{max} observed were very close when ketotifen and UDPGA varied (table 2).

Glucuronidation by Microsomes from Different Human Livers. Results are shown in table 3. No significant difference was observed between sexes. Solubilization by Triton enhanced the *N*-glucuronidation rate about 3-fold, irrespective of the sex.

Inhibition of *N*-Glucuronidation by Bilirubin and 4-Nitrophenol. The two K_M observed with ketotifen might suggest the presence of two isoenzymes. So, two well known substrates of UDP-glucuronyltransferase, namely, bilirubin and 4-nitrophenol, were tested for their ability to inhibit the *N*-glucuronidation of ketoti-

TABLE 2

Kinetic constants of ketotifen N-glucuronidation by human liver microsomes

For K_M determination of ketotifen, UDPGA was 5 mM; for K_M determination of UDPGA, ketotifen was 0.19 mM. Incubations were carried out for 30 min with 2.2 and 1.5 mg of protein, respectively, for native and detergent-treated microsomes.

Substrate	K_M		V_{max}	
	Native microsomes	Detergent-treated microsomes	Native microsomes	Detergent-treated microsomes
	μM		pmol × min^{-1} × mg^{-1} protein	
Ketotifen	12.5	42	19	125
	100		50	
UDPGA	250	440	43	118

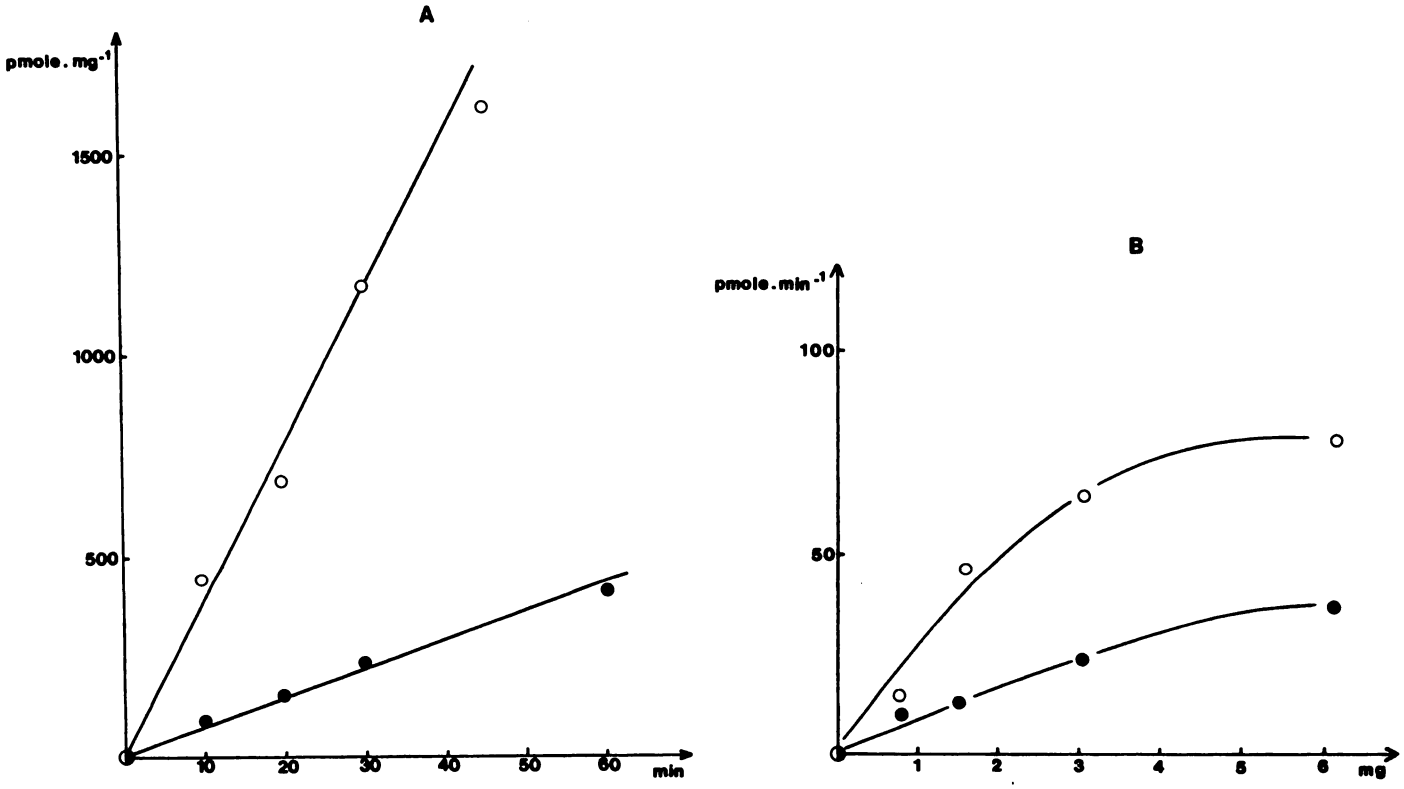

FIG. 2. *Effect of time of incubation and protein concentration on ketotifen N-quaternary glucuronide formation.*

Incubations were carried out with 5 mM UDPGA and 0.07 mM ketotifen. *A*, incubations were conducted with 3 mg protein for native microsomes (●) or Triton X-100-deterged microsomes (○). Results are expressed as pmol ketotifen conjugated by 1 mg microsomal protein. *B*, incubations were carried out for 30 min with native (●) or Triton X-100-deterged microsomes (○). Results are expressed as pmol ketotifen conjugated/minute.

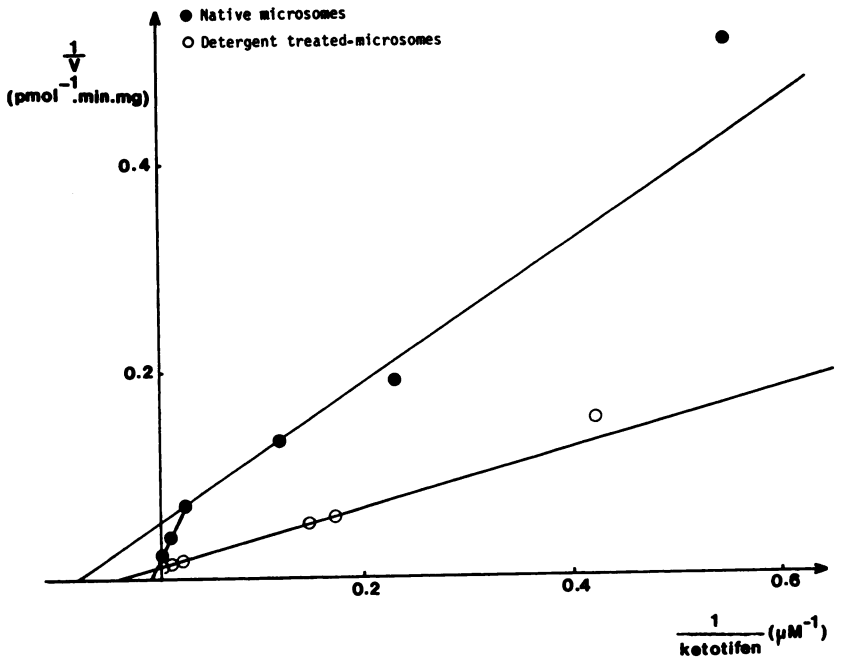

FIG. 3. *Double reciprocal plot of ketotifen of N-glucuronidation by human liver microsomes.*

Incubations were carried out for 30 min with 5 mM UDPGA and 2.2 mg protein for native microsomes (●) and 1.5 mg protein for detergent-treated microsomes (○). Velocity is expressed as pmol ketotifen conjugated in 1 min by 1 mg protein and ketotifen concentration in μM.

TABLE 3

N-Glucuronidation rate of ketotifen by human liver microsomes

Results are expressed as pmol × min^{-1} × mg^{-1} protein, the mean ± SD when three or more determinations were performed or individual values when only two determinations were carried out. Incubations were conducted for 30 min, with 0.1 mM ketotifen, 5 mM UDPGA, 1.7 to 2.6 mg of proteins for native microsomes, and 1.2 to 1.8 for detergent-treated microsomes.

	Men	Women
Natives microsomes	33 ± 14 (4)	36 ± 7 (3)
Detergent-treated microsomes	98 ± 30 (3)	61–90 (2)
Fold-up increase due to detergent	3.3 ± 0.5 (3)	2.1–2.4 (2)

fen. These two substrates were added to the incubation medium at a final concentration corresponding to their respective K_M for human glucuronyltransferase (14, 15). Under these conditions, neither bilirubin nor 4-nitrophenol inhibited the *N*-glucuronidation of ketotifen: values of 115 and 116 pmol·min^{-1}·mg^{-1} protein, respectively, were obtained vs. 122 for control incubations (*i.e.* in the presence of sodium hydroxide).

Discussion

The purpose of the present study was to investigate *in vitro* with human liver microsomes, the metabolic pathways of ketotifen and particularly the *N*-glucuronidation, a novel route of biotransformation.

The present results, compared with those obtained *in vivo* in man, allow the authors to draw the following conclusions. 1) Common metabolites were found *in vitro* with human liver microsomes and *in vivo* in human urine, namely nor-ketotifen, N-oxide, and *N*-glucuroketotifen. 2) Two additional metabolites, the reduced ketotifen derivative and its glucuronide, were recovered in human urine but were not found *in vitro* under the described incubation conditions. This could be due to the intracellular location of ketone reductase, a cytosolic enzyme (16) which is virtually absent in microsomal preparations. In microsomes, reduction may occur under partial anaerobic conditions, a protocol which was not investigated in the present study. 3) The *N*-glucuroketotifen was only formed in the presence of UDGPA irrespective of the presence of a NADPH-generating system. This indicates that a prior biotransformation of ketotifen is not necessary for glucuronidation. Such results obtained *in vitro* confirm the possibility of forming a glucuronide of a tertiary amine as already shown *in vivo* with cyproheptadine, tripelennamine, cyclobenzaprine, LY 108 380, and ketotifen (1–5). However, among the investigated animal species, this typical and unusual conjugation reaction was not observed (dog, rat) or was a minor route (rabbit, rhesus monkey, baboon) (5, 7). So, human liver microsomes were the only appropriate material to characterize this enzymatic reaction.

The determination of the kinetic parameters of the ketotifen *N*-glucuronidation in native microsomes showed two apparent affinities for ketotifen, but only one for UDPGA. These two different K_M values for ketotifen may reflect the presence of two isoenzymes of UDP-glucuronyltransferase. This hypothesis may be supported by the data of Mahu *et al.* indicating that in human liver, as in rat liver, UDP-glucuronyltransferase is heterogeneous (15). A most likely explanation is based on the accessibility of the microsomal UDP-glucuronyltransferase for ketotifen: the local concentration of the substrate may greatly vary and thus affect the apparent affinity of the enzyme, in relation to its transverse location in the endoplasmic reticulum membrane. This hypothesis is strengthened by the observation of only one K_M value for ketotifen in detergent-treated microsomes: in that case, enzyme molecules are not embedded in the membrane, but are equally accessible to the substrate and thus exhibit only one apparent affinity constant. In

addition, the V_{max} value is markedly increased by detergent solubilization, indicating that a larger number of enzyme molecules were fully active. These values of K_M of human liver microsomes for ketotifen and UDPGA are close to those reported previously for 4-nitrophenol and UDPGA by Mahu *et al.*, whereas the V_{max} measured with both native and detergent-treated microsomes were low compared with generally reported V_{max} values (11, 15). On the other hand, the stimulation of the V_{max} by Triton X-100 was comparable to those found by other authors (15) and quite similar from liver to liver (table 3). No sex difference for the *N*-glucuronidation of ketotifen was observed, as it has already been reported for some other drug-metabolizing enzymes from human liver (12).

In rat, bilirubin and 4-nitrophenol are the well known substrates of two UDP-glucuronyltransferase isoenzymes, induced, respectively, by phenobarbital and 3-methylcholanthrene (8, 9). In man, Mahu *et al.* (15) drew the conclusion of a likely heterogeneity of UDP-glucuronyltransferase using these two substrates as models. In the present investigation, none of these compounds was able to diminish the glucuronidation rate of ketotifen under our experimental conditions. With rat microsomes, by use of a similar procedure, 4-nitrophenol at a concentration close to its K_M, has been shown to partially inhibit bilirubin (17) and methylumbelliferone (18) conjugation, and conversely, bilirubin, at a concentration roughly close to its K_M, inhibits 4-nitrophenol conjugation (17). These observations, added to the previous finding indicating that man is the sole species able to form *N*-quaternary glucuronide in appreciable amounts, strengthen the assumption that a specific UDP-glucuronyltransferase isoenzyme might be involved in the *N*-glucuronidation reaction. Further studies are now in progress to verify this assumption.

In conclusion, the biotransformation of ketotifen by human liver microsomes exhibited three of the four metabolic pathways observed *in vivo* in man. The reduction pathway, which was absent in the *in vitro* study, is likely to implicate cytosolic enzymes.

Human liver microsomes are a useful and suitable *in vitro* model for studying drug metabolism in man. The advantage of the system which is of major interest is its predictive value regarding the human species. In particular, for investigation of unusual metabolic routes—as in the case of the *N*-glucuronidation of ketotifen—the system demonstrates incomparable advantages. However, the results obtained, as shown by the case of the reduction of the ketotifen, reflect only partially the multiplicity of events in the *in vivo* systems; this has to be considered carefully in the interpretation of *in vitro* data.

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