THE PHASE I BIOTRANSFORMATION OF THE POTENTIAL ANTILEUKOTRIENIC DRUG QUINLUKAST IN RAT MICROSOMES AND HEPATOCYTES

Vladimír Wsól[a1,b,]*, Barbora Szotáková[a2,b], Vendula Baliharová[b1], Luděk Šišpera[b2], Michal Holčapek[c1], Lenka Kolářová[c2], Bohumila Suchanová[b3], Miroslav Kuchař[d] and Lenka Skálová[a3,b]

[a] *Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic; e-mail:* [1] *wsol@faf.cuni.cz,* [2] *szotako@faf.cuni.cz,* [3] *skaloval@faf.cuni.cz*

[b] *Research Centre LN00B125, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic; e-mail:* [1] *baliharova@faf.cuni.cz,* [2] *sispera@faf.cuni.cz,* [3] *suchanova@faf.cuni.cz*

[c] *Department of Analytical Chemistry, Faculty of Chemical Technology, University of Pardubice, nám. Čs. legií 565, 532 10 Pardubice, Czech Republic; e-mail:* [1] *michal.holcapek@upce.cz,* [2] *lenka.kolarova@upce.cz*

[d] *Research Institute for Pharmacy and Biochemistry, U Kabelovny 130, 102 37 Prague 10, Czech Republic; e-mail: miroslav.kuchar@zentiva.cz*

Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

The phase I metabolism of quinlukast (VÚFB 19363, Q; 4-{[4-(2-quinolylmethoxy)phenyl]-sulfanyl}benzoic acid), a new antiasthmatic drug with significant antileukotriene effects, was investigated in rat microsomes and hepatocytes. Quinlukast, incubated with rat liver microsomal fraction under oxidative conditions, generated four metabolites, M2–M5. Based on comparison with synthetically prepared standards, metabolites M2 and M4 were identified as sulfoxide and sulfone of the parent compound, respectively. Metabolites M3 and M5 were identified as quinlukast dihydrodiols. For all the metabolites the apparent kinetic parameters K'_m, V'_{max} and metabolic efficiency Cl_{int} were calculated. No metabolite was found in rat liver cytosol. In vitro studies with primary cultures of isolated hepatocytes were designed to evaluate time dependent (2, 4, 8 and 24 h) and concentration dependent (0.005–0.1 mmol/l) formation of metabolites of quinlukast. Four metabolites were detected in culture medium. Three of them were identical to metabolites found in incubation of quinlukast with microsomes (M2, M3 and M5) and another, the most polar metabolite, M1, was detected. The basic metabolic pathways were proposed for quinlukast in rats.

Keywords: Antiasthmatics; Drug metabolism; Metabolic pathways; Hepatocytes; Microsomes; Enzyme kinetics; Kinetic parameters; LC-MS; Biotransformations; Antihistaminics; Metabolites.

Antileukotriene drugs inhibit the formation or action of leukotrienes, which are potent lipid mediators. Leukotrienes LTB_4 and cysteine-containing leukotrienes (Cys-LT), LTC_4, LTD_4 and LTE_4, originate from oxidative metabolism of arachidonic acid through a key enzyme, 5-lipoxygenase, in lung tissue and in a number of inflammatory cells[1]. It is now widely recognized that Cys-LTs play an important role in asthma, participating both in the bronchoconstriction and in the chronic inflammatory component of the disease[2]. In human tissues, effects of these leukotrienes are mediated by activation of $Cys-LT_1$ receptors. Consequently, the number of structurally different $Cys-LT_1$ antagonists has been expanding[3] in the last years and some are already available for clinical use (zafirlukast, pranlukast and montelukast)[4,5].

New original compounds – derivatives of (arylsulfanyl)benzoic acid – with significant antileukotrienic effects were prepared in the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Of them, quinlukast (VÚFB 19363, Q), 4-{[4-(2-quinolylmethoxy)phenyl]sulfanyl}-benzoic acid (Fig. 1), is considered to be the most promising[6].

The IC_{50} value for Q in inhibition of binding to leukotriene D_4 receptors is comparable to montelukast and zafirlukast, while leukotriene B_4 biosynthesis inhibition by Q is much more effective than that of montelukast and zafirlukast (*manuscript in preparation*). The main advantage of Q is its multiple antileukotrienic, anti-inflammatory and antiasthmatic effect.

With respect to promising biological activities further pre-clinical trials of Q have been initiated. Biotransformation study represents an integral and important part of development of each new drug. The study described in this paper was designed to characterize the phase I in vitro metabolism of Q in rats and to identify the principal Q metabolites. The kinetics of metabolites formation was studied in primary cultures of rat hepatocytes and in subcellular fractions of rat liver homogenate.
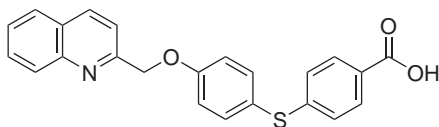

Fig. 1
Chemical structure of quinlukast

EXPERIMENTAL

Chemicals

Quinlukast (VÚFB 19363, Q) and its potential metabolites, Q sulfoxide and Q sulfone, were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Enzymatic tests were performed using NADPH from Sigma–Aldrich (Prague, Czech Republic). Acetonitrile (MeCN), methanol (MeOH) and dimethyl sulfoxide (DMSO) (all HPLC grade) were obtained from Riedel–deHaen (Seelze, Germany). Collagenase was purchased from Sevapharma (Prague, Czech Republic). The Ham F12 medium, William's E medium, foetal calf serum, antibiotics, insulin and collagen were supplied by Sigma–Aldrich (Prague, Czech Republic). All other chemicals were of the highest purity commercially available.

Animals

Male Wistar rats (10–12 weeks) were obtained from the BioTest (Konárovice, Czech Republic). They were kept on standard rat food with free access to tap water, in animal quarters under a 12-h light-dark cycle. Rats were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals (Protection of Animals from Cruelty Act. No. 246/92, Czech Republic).

Isolation of Microsomal and Cytosolic Fractions

Livers of male Wistar rats (10–12 weeks) were used as source of cytosol and microsomes. Livers were homogenized in 0.1 M Na phosphate buffer, pH 7.4. The microsomal and cytosolic fractions were obtained by fractional ultracentrifugation of the homogenate[7]. A rewashing step (followed by second ultracentrifugation) was added at the end of the microsomes preparation procedure. Microsomes were finally resuspended in the homogenization buffer containing 20% glycerol (v/v) and stored at –80 °C. The protein content was determined according to Lowry with 0.1% SDS [8].

Isolation of Hepatocytes

Hepatocytes were obtained by the two-step collagenase method[9]. In the first step, the liver was washed with 150–200 ml of a solution without calcium, with the aim to remove the rest of blood and make the cell–cell junction weaker. In the second step, hepatocytes were released by action of collagenase (50 mg/100 ml) in perfusion solution with calcium. Second perfusion took 5–6 min (recirculation system). Isolated hepatocytes were washed three times and mixed with culture medium. The culture medium consisted of a 1:1 mixture of Ham F12 and Williams' E, supplemented as described earlier[10,11]. The viabilities of cells measured by Trypan Blue staining according to the Sigma protocol were 85–90%. Three million of viable cells in 3 ml of culture medium were placed into 60-mm plastic dishes precoated with collagen. The foetal calf serum was added in culture medium (5%) to promote cell attachment during the first four hours after plating. Then the medium was exchanged for fresh one without serum. The cultures were maintained at 37 °C in a humid atmosphere with 5% CO_2.

Cytotoxicity Assay

Cytotoxicity was assayed by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) test as described[12]. Cells, seeded in 96-well plates, were incubated with Q at different concentrations (0.005–0.1 mmol/l) for 24 h, followed by incubation with MTT. An amount of 25 µl of a MTT solution in phosphate buffer (3 mg/ml) per well was applied without removal of the medium; after 2.5 h the medium was discarded and cells were lysed with 0.08 M HCl in propan-2-ol (50 µl/well). Absorbance of the formazane product was measured at 595 nm. The absorbance of formazane in cells treated with Q was compared to that in control cells exposed to medium with 0.5% DMSO alone.

Incubation of Microsomal and Cytosolic Fractions with Quinlukast

A liver microsomal suspension (50 µl corresponding to 50 mg of wet tissue) was incubated in Eppendorf microtubes with quinlukast as substrate (final concentrations ranging from 0.01 to 1 mmol/l) and 0.5 mM NADPH or NADH in a total buffer volume of 0.3 ml. The liver cytosolic fraction (50 µl corresponding to 7 mg of wet tissue) was incubated in Eppendorf microtubes with quinlukast as substrate (final concentrations 0.05, 0.2 and 0.5 mmol/l) and 0.5 mM NADPH in a total buffer volume of 0.3 ml. Incubations were performed for 30 min at 37 °C. All incubations were terminated by cooling to 0 °C and addition of 10 µl of 1 M HCl. Quinlukast and its metabolites were extracted twice with two volumes of ethyl acetate–*n*-heptane mixture (1:3, v/v), and the combined extracts were evaporated to dryness in vacuum. The dry samples were dissolved in 5 mM phosphate buffer, pH 7.0, prior to their HPLC injection.

Incubation of Hepatocytes Primary Culture with Quinlukast

A stock solution of Q dissolved in DMSO was added to fresh medium. The final concentration of DMSO in medium was 0.1%.

Hepatocyte monolayers (18–24 h after isolation) were incubated with 50 or 100 µM Q. Aliquots of the medium (0.5 ml) were collected in the intervals of 0, 2, 4, 8 and 24 h. For kinetic study various concentrations (0, 5, 10, 25, 50, 75, 100 µmol/l) of the substrate were used and incubation proceeded for 24 h. All medium samples were stored frozen at –80 °C prior to their solid-phase extraction and HPLC analyses.

Solid-Phase Extraction

SPE of the hepatocyte samples were performed on Sep-Pak Light tC18 cartridges (145 mg sorbent; Waters, Prague, Czech Republic). The cartridges were conditioned with methanol (2 ml) and a washing solution, 10 mM phosphate buffer (2 ml, pH 7.0), before applying the samples. Hepatocyte samples (500 µl) prepared as described above were manually loaded onto the cartridges which were purged with washing solution (4.5 ml). Finally, all SPE cartridges were eluted with 1 ml of methanol. The eluting solvents were evaporated, and dry samples reconstituted in 120 µl of solution consisting of 5 mM phosphate buffer, pH 7.0, in MeCN–H_2O (40:60, v/v) and prepared for HPLC injection.

HPLC Assay for Quinlukast and Its Metabolites

Quinlukast and its metabolites were measured with an HPLC system consisting of an LC-10 AD$_{VP}$ gradient pump, SIL-10 AD$_{VP}$ autoinjector, FCV-10 AL$_{VP}$ solvent mixer, CTO-10 AC$_{VP}$ column oven, SCL-10 A$_{VP}$ system controller and SPD-M10 A$_{VP}$ UV-VIS photodiode array detector with detection set at 220–300 nm range (285 nm for metabolites). Data from these chromatographic runs were processed using the Chromatography Laboratory Automated Software System Class VP (version 6.12) from Shimadzu (Prague, Czech Republic). The assay was performed on a Purospher RP18e analytical column (5 μm, 125 × 3 mm; Merck, Prague, Czech Republic) equipped with a Hypersil BDS C18 guard column (5 μm, 4 × 4 mm; Agilent, Prague, Czech Republic). The HPLC method involved the following gradient system using 8 mM H$_3$PO$_4$ in H$_2$O–MeOH–MeCN (70:16.5:13.5, v/v/v) as mobile phase A and 8 mM H$_3$PO$_4$ in H$_2$O–MeOH–MeCN (10:49.5:40.5, v/v/v) as mobile phase B. The flow rate was set at 0.7 ml/min. From 0 to 13 min, the ratio of mobile phase A to B was linearly changed from 73:27 to 51:49. Mobile phase B was then increased to 80% at 13.5 min. Mobile phase B was held at 80% for 6.5 min, after which it was reverted back to 27% at 20.5 min. The equilibration time was 15.5 min. All chromatographic runs were performed at 25 °C. The blank medium spiked with M2 was used for calibration. Weighted linear regression analysis was performed by plotting the peak area versus analyte concentration (0.08–8.00 μmol/l). Regression line parameters were used to calculate concentrations of other metabolites in all biological samples. The limit of reliable quantification (coefficient of variation, CV ≤ 20%) was taken as 80 nmol/l for all three metabolites. Intra-day variability was assessed by quadruple analyses of incubated samples. The CV values did not exceed 4.9% (M2), 5.1% (M3) and 7.2% (M5). Absolute recovery of M2 from medium was 84.7 ± 2.6% at the 2.4 μmol/l level. Figure 2 illustrates the resolution of Q and its metabolites in male rat hepatocytes.
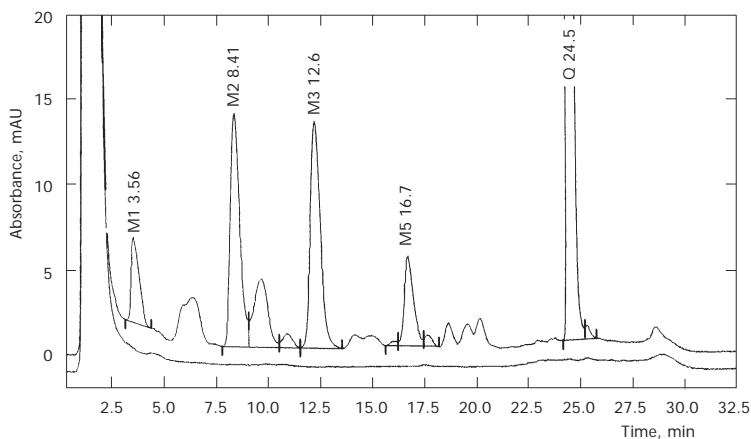

FIG. 2
HPLC chromatogram of the rat hepatocyte medium. 24-h incubation with 100 μM Q as substrate is shown together with blank sample of hepatocytes. HPLC conditions are described in Experimental

HPLC/MS

The equipment used consisted of a Model 616 pump with a quaternary gradient system, a Model 996 diode-array UV detector, a Model 717+ autosampler, a thermostatted column compartment and a Millennium chromatography manager (all from Waters, Milford, MA, U.S.A.). The UV detector outlet of the liquid chromatograph was connected to the ion trap analyser Esquire3000 (Bruker Daltonics, Bremen, Germany) using electrospray ionization (ESI) in both the positive- and negative-ion mode. Data were acquired in the mass range *m/z* 50–1000. The pressure of the nebulising gas was 414 kPa, the flow rate and temperature of the drying gas was 11 l/min and 365 °C, respectively. The ion trap analyser was tuned to give an optimum response for *m/z* 400. The isolation width for MS/MS experiments was *m/z* 4, and the collision amplitude was 1 V. The compound stability was 100% (first experiments) and 20%.

RESULTS

Metabolism of Quinlukast in Rat Microsomes and Cytosol

Q in a saturated solution (0.5 mmol/l) was incubated with microsomes under aerobic or anaerobic conditions. NADH or NADPH was used as a coenzyme. Composition of the incubation mixture was analysed by HPLC. Four metabolites (M2–M5) were detected in the incubation mixture after aerobic incubation with NADPH. When NADH coenzyme was used substantially lower amounts of metabolites were formed. Only a negligible amount of metabolites was found in samples incubated under anaerobic conditions (data are not shown). Metabolites M2 and M4 were compared with synthetically prepared standards and identified as Q sulfoxide and Q sulfone, respectively. While Q sulfoxide was the principal metabolite of Q in rat microsomes, Q sulfone was only a minor one the concentration of which was near the limit of detection. Using HPLC/MS with positive-ion ESI, the first-stage mass spectra of Q and of all four discussed metabolites showed protonated molecules without any fragmentation, which enabled an easy molecular weight determination (see the spectrum of M3 in Fig. 3a). Following MS/MS spectra of [M + H]$^+$ ions (Fig. 3b) yielded the characteristic fragment ions supporting the structure assignment of M3 and M5. The mass spectra of M3 and M5 differ solely in the relative abundances of some fragment ions in MS/MS spectra, hence only one example illustrates an identification (Fig. 3). Cleavage of a bond between the sulfur atom and the benzene ring leads to the *m/z* 269 ion and subsequent neutral loss of water corresponds to the *m/z* 251 ion. Similarly, the cleavage of the bond between the etheric oxygen and the CH$_2$ group leads to the *m/z* 177 ion and subsequent loss of water corresponds to the *m/z* 159 ion. Two molecules of water

are readily lost from the $[M + H]^+$ ion (the fragment ions *m/z* 404 and 386), which is typical of vicinal hydroxy groups.

Kinetic studies were performed under aerobic conditions with NADPH as coenzyme and substrate concentrations 0.01–1.0 mmol/l. Concentrations of metabolites M2, M3 and M5 were measured. The curves obtained are presented in Fig. 4. Kinetic parameters – apparent K'_m and V'_{max} and metabolic efficiency Cl_{int} (defined as V_{max}/K_m ratio) – were calculated using GraphPad Prism 3.0 software. Data are shown in Table I.

Q (concentrations 0.05, 0.2 and 0.5 mmol/l) was incubated with rat cytosol under aerobic or anaerobic conditions. NADH or NADPH was used as a coenzyme. No metabolites were detected in incubation mixtures (data are not shown).
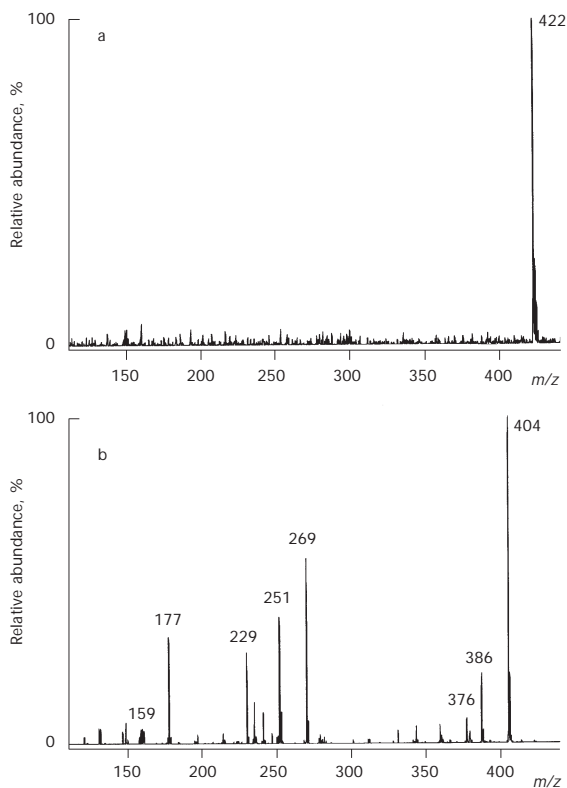

Fig. 3
ESI-MS spectra of quinlukast metabolites M3: a full scan, b MS/MS spectrum

TABLE I

Apparent kinetic parameters of the respective biotransformation enzymes in rat liver microsomal fraction

Metabolite	V'_{max}	K'_m	Cl_{int}
M2	1820 ± 54	18.8 ± 2.6	96.8
M3	271 ± 6	13.8 ± 1.7	19.6
M5	321 ± 8	14.2 ± 1.9	22.6

The values represent the average of 4–6 determinations. V'_{max} in pmol/30 min (per mg of protein), K'_m in µmol/l, Cl_{int} (metabolic efficiency, defined as V_{max}/K_m ratio) in µl/30 min (per mg of protein).
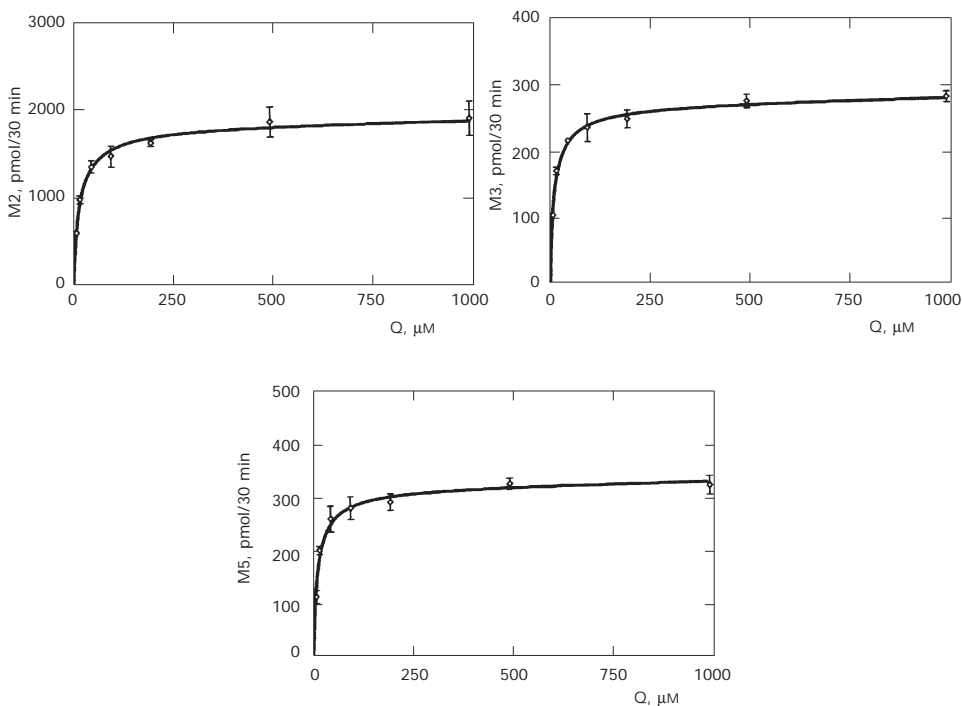

FIG. 4

Rate of formation of Q metabolites versus quinlukast concentration in rat liver microsomes. Each point represents the average and S.E.M. of 4–6 determinations. Data are normalized for 1 mg of protein

Metabolism of Quinlukast in Rat Hepatocytes

Primary cultures of rat hepatocytes were incubated with 0.05 or 0.1 mM Q and aliquots of the medium were collected and analysed. Four metabolites were detected in culture medium. Three of them were identical to metabolites found in incubation of Q with microsomes. No Q sulfone, a minor metabolite observed in microsomes, was detected in culture medium. On the other hand, another metabolite (M1) was detected in hepatocyte medium. Based on polarity and spectra analysis, M1 seems to be a conjugate of Q or its metabolite. Its structure and kinetics will be under study in the next project concerning phase II of Q metabolism.

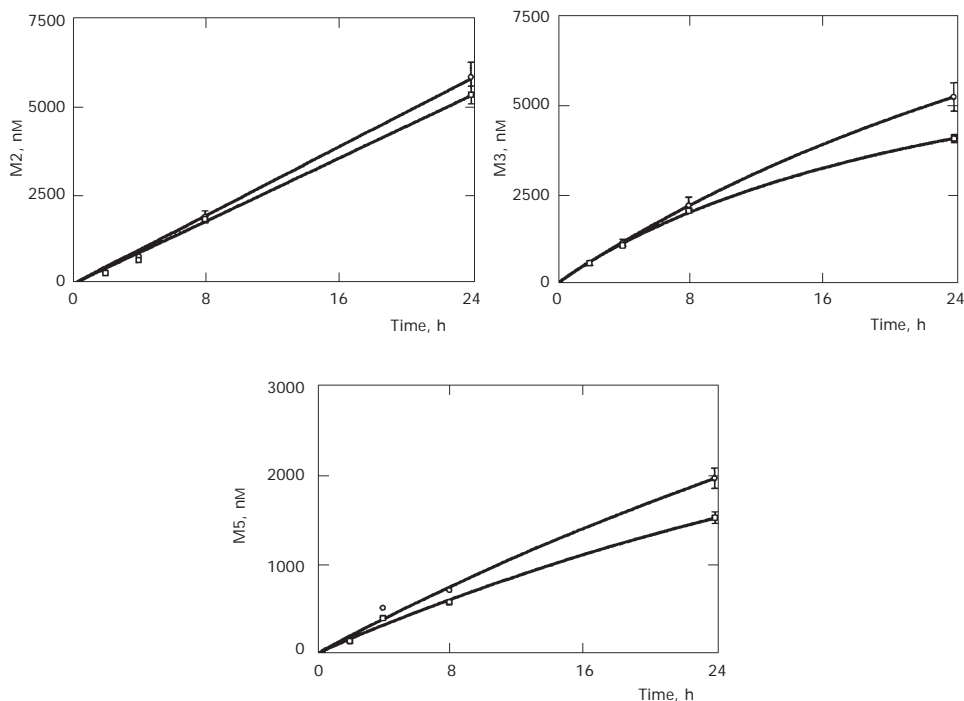The time-dependent (0, 2, 4, 8 and 24 h) formation of metabolites is presented in Fig. 5.

Fig. 5

Time dependent (0, 2, 4, 8 and 24 h) formation of M1, M2, M3 and M5 in culture of the rat hepatocyte medium for two concentrations of Q (50 (□) and 100 (○) µmol/l). Each point represents the average and S.E.M. of 4–6 determinations. Data are normalized for 1 million of cells

For kinetic studies, various concentrations of substrate (0.005–0.1 mmol/l) were used. Concentrations of metabolites M1, M2, M3 and M5 were measured in culture medium after 24-h incubation. The curves obtained are presented in Fig. 6. Kinetic parameters – apparent K'_m and V'_{max} – together with Hill coefficient were calculated using GraphPad Prism 3.0 software. Kinetic parameters obtained served for calculation of metabolic efficiency Cl_{int}. Data are shown in Table II.

Q was shown to be non-cytotoxic in the concentration range tested (0.005–0.1 mmol/l) in primary cultures of rat hepatocytes.
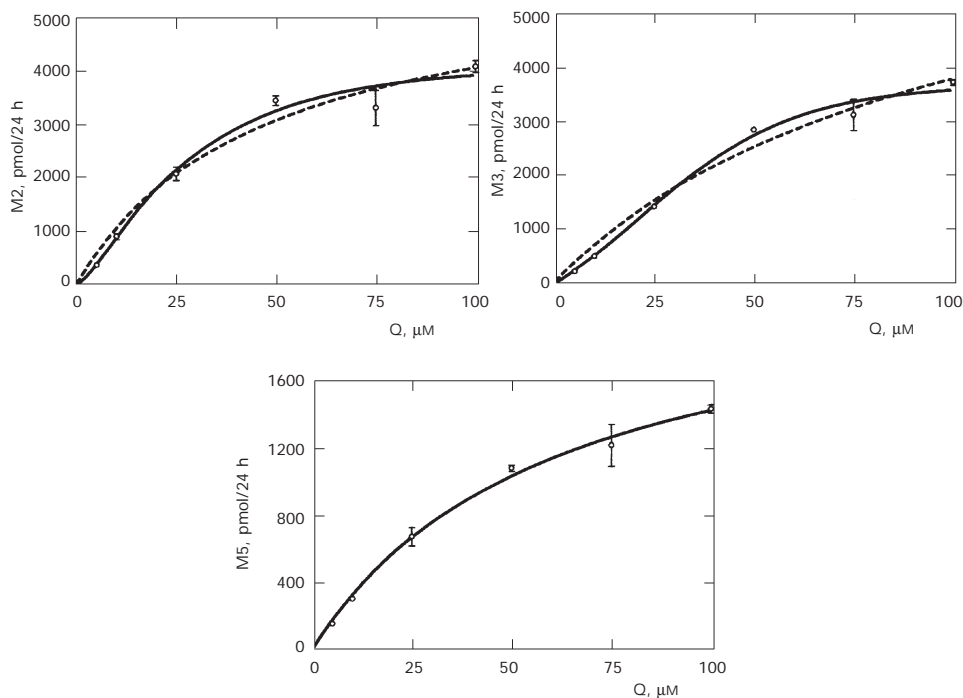

FIG. 6

Rate of formation of Q metabolites versus quinlukast concentration after 24-h incubation in 1 ml of culture medium. The dotted line indicates the Michaelis–Menten kinetics and the full line shows a better fit for sigmoidal dose-response kinetics. Each point represents the average and S.E.M. of 4-6 determinations. Data are normalized for 1 million of cells

DISCUSSION

Recently, several Cys-LT1 receptor antagonists are available for clinical management of asthma. Their metabolic pathways have been studied and ascertained. Dicarboxylic acid, acyl glucuronide, sulfoxides, hydroxylated and methylhydroxylated metabolites are the montelukast metabolites formed in humans[5,13]. Biotransformation of zafirlukast in rats, dogs and mice includes hydrolysis, hydroxylation and demethylation[14]. Hydroxylation, sulfur oxidation and conjugation of verlukast with glucuronic acid and glutathion were reported[15,16]. The identification of the main metabolic pathways of new potential antileukotrienic drug Q was necessary for starting its pharmacokinetic tests and clinical trials.

Two in vitro models were used for this purpose: subcellular fractions (microsomes and cytosol) of rat liver homogenate and primary cultures of rat hepatocytes. Four metabolites were revealed in rat microsomes. Q sulfoxide represented the principle one. A relatively high metabolic efficiency Cl_{int} indicates high metabolic efficiency of sulfur oxidation of Q. Similarly to verlukast[17], also in Q oxygen is incorporated into the structure in the form of relatively unstable Q epoxides. These epoxides are then very quickly converted probably by epoxide hydrolase to the corresponding Q dihydrodiols (metabolites M3 and M5). The mass of these Q dihydrodiols was confirmed by mass spectrometry (MS and MS2). The position of hydroxyl groups was proposed on the basis of metabolic similarity with verlukast[17] and taking into account different retention times in the HPLC system used. Just M5 is probably able to form a five-membered ring that

TABLE II
Apparent kinetic parameters of the respective biotransformation enzymes in rat hepatocyte medium

Metabolite	V'_{max}	K'_m	Cl_{int}	Hill coeff.
M2	4435 ± 431	26.0 ± 3.3	170.6	1.48 ± 0.27
M3	4239 ± 390	36.3 ± 4.6	116.8	1.68 ± 0.23
M5	1915 ± 180	42.2 ± 5.1	45.4	1.16 ± 0.17

The values represent the average of 4–6 determinations. V'_{max} in pmol/24 h (per million of cells), K'_m in µmol/l, Cl_{int} (metabolic efficiency, defined as V_{max}/K_m ratio) in µl/24 h (per million of cells).

produces less hydrogen bonds with mobile phase, which can be explanation for its higher retention time in comparison to the other dihydrodiol M3. As only negligible amount of Q sulfone was formed, structure of Q probably defended sulfur atom against second oxidation. Finding no metabolites in liver cytosol corresponded to our expectation of no reductive or hydrolytic transformation of Q.

In primary cultures of rat hepatocytes, four metabolites were also detected. Three of them were identical to metabolites found in incubation of Q with microsomes. Q sulfone, a minor metabolite observed in microsomes, was not found in hepatocytes probably due to a substantially lower concentration of the substrate used in hepatocytes than in microsomes. On the other hand, another metabolite (M1) was detected in hepatocyte medium. Based on polarity of the HPLC system and spectral analysis ([M + H]$^+$ ion m/z 515), M1 seems to be a conjugate of Q or Q metabolite. While in microsomes the formation of metabolites M3 and M5 was 5–8 less extensive than sulfur oxidation of Q, the amount of M3 detected in hepatocyte medium was similar to the amount of Q sulfoxide. Comparing the two models used – microsomes and hepatocytes – several factors might shift proportion of metabolites: secondary metabolic transformation, stability and orientation of enzymes, cooperation of enzymes, accessibility of substrate etc.[11].

Concentration of all metabolites increased during 24-h Q incubation in hepatocytes. While formation of Q sulfoxide was linear up to 24 h (in addition, the chemical stability during the 24-h incubation was confirmed), metabolites M1, M3 and M5 increased linearly only for 4–8 h. Exhaustion of substrate, damage of enzymes or secondary metabolic transformation might affect metabolite concentration detected in medium. Chemical stability during the 24-h incubation for M1, M3 and M5 was not measured because these metabolites were not available as standards.

No or low differences between concentration of metabolites formed in hepatocytes incubated with 0.05 and 0.1 mM Q was observed in all the time intervals tested. This finding was in accordance with low values of apparent K_m calculated from kinetic curves for all metabolites. While the shape of kinetic curves for M2, M3 and M5 in microsomes corresponded well with the Michaelis–Menten kinetics, certain sigmoidal deformation of curves was observed for these metabolites in hepatocyte culture. For this reason, two fittings of experimental points were made: first corresponding to the Michaelis–Menten kinetics and second corresponding to the sigmoidal dose-response kinetics. Hill coefficients 1.5 and 1.7 characterized the formation of metabolites M2 and M3, respectively, in primary cultures of rat

hepatocytes. This finding can either be due to partial cooperation of enzyme systems in their formation or to a contribution of transport proteins in their release into medium.

In conclusion, the in vitro study of Q metabolism revealed five Q metabolites in rat. Based on our results the metabolic pathways for Q in rat liver microsomes and hepatocytes were proposed (Fig. 7). Phase I metabolites were identified (M2 and M4) or their structures were suggested on the basis of ESI-MS spectra (M3 and M5), and kinetic parameters of their in vitro formation were calculated and evaluated.
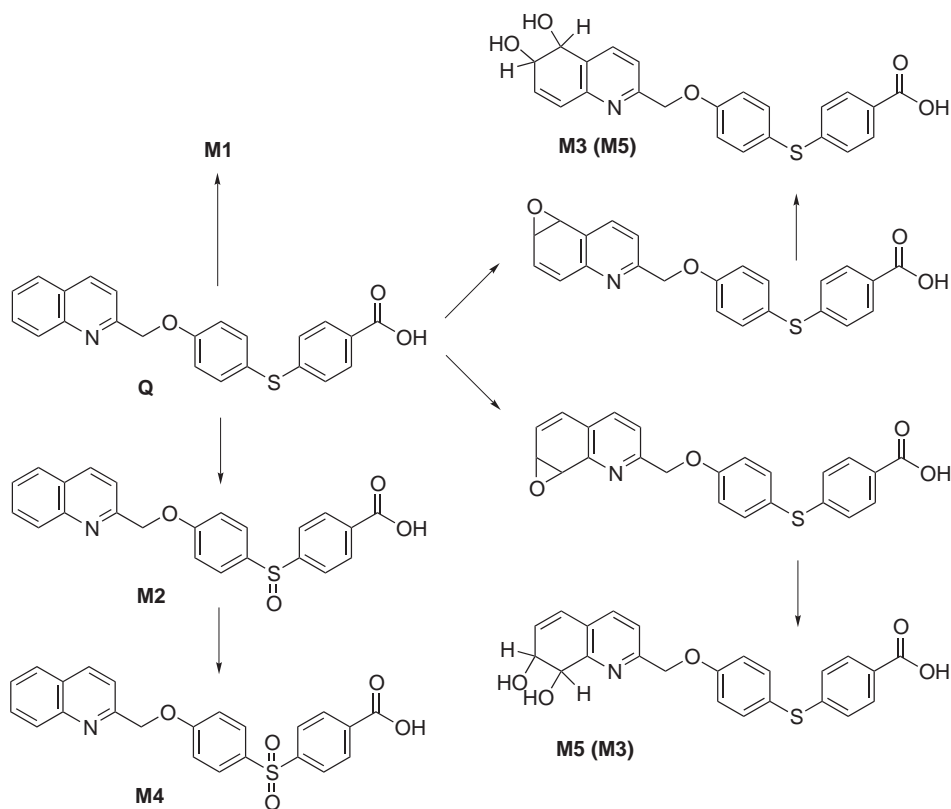

FIG. 7
Proposed biotransformation pathways for quinlukast in rat liver microsomes and hepatocytes

REFERENCES

1. Dahlen B., Hedquist S., Hammarstrom S., Samuelsson B.: *Nature (London)* **1980**, *288*, 484.
2. Jain N. K., Kulkarni S. K., Singh A.: *Eur. J. Pharmacol.* **2001**, *423*, 85.
3. Claesson H. E., Dahlen B.: *J. Intern. Med.* **1999**, *245*, 205.
4. Chung K. F., Barnes P. J.: *Drugs Today* **1998**, *34*, 375.
5. Chiba M., Xu X., Nishime J. A., Balani S. K., Lin J. H.: *Drug Metab. Dispos.* **1997**, *25*, 1022.
6. Kuchar M., Kmonicek V., Panajotova V., Brunova B., Jandera A., Jirickova H., Bucharova V.: U.S. 6,303,612 B1, 2001; *Chem. Abstr.* **2000**, *132*, 49797.
7. Gillette J. R.: *Fundamentals of Drug Metabolism and Drug Disposition*, p. 400. The Williams and Wilkins, Baltimore 1971.
8. Markwell M. A., Haas S. M., Bieber L. L., Tolbert N. E.: *Anal. Biochem.* **1978**, *87, 206*.
9. Berry M. N., Edwards A. M., Barritt G. J.: *Laboratory Techniques in Biochemistry and Molecular Biology*, pp. 1–470. Elsevier Science, Amsterdam 1991.
10. Isom H. C., Georgoff I.: *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *8*, 6378.
11. Maurel P.: *Adv. Drug Deliv. Rev.* **1996**, *22*, 105.
12. Denizat F., Lang R.: *J. Immunol. Methods* **1986**, *89*, 271.
13. Balani S. K., Xu X., Pratha V., Koss M. A., Amin R. D., Dufresne C., Miller R. R., Arison B. H., Doss G. A., Chiba M., Freeman A., Holland S. D., Schwartz J. I., Lasseter K. C., Gertz B. J., Isenberg J. I., Rogers J. D., Lin J. H., Baillie T. A.: *Drug Metab. Dispos.* **1997**, *25*, 1282.
14. Savidge R. D., Bui K. H., Birmingham B. K., Morse J. L., Spreen R. C.: *Drug Metab. Dispos.* **1998**, *26*, 1069.
15. Nicol-Griffith D. A., Yergey J., Trimble L., Williams H., Rasori R., Zamboni R.: *Drug Metab. Dispos.* **1992**, *20*, 383.
16. Nicol-Griffith D. A., Gupta N., Twa S. P., Williams H., Trimble L., Yergey J. A.: *Drug. Metab. Dispos.* **1995**, *23*, 1085.
17. Grossman S. J., Herold E. G., Drey J. M., Alberts D. W., Umbenhauer D. R., Patrick D. H., Nicoll-Griffith D., Chauret N., Yergey J. A.: *Drug Metab. Dispos.* **1993**, *21*, 1029.