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# The stereoselective biotransformation of the anti-obesity drug sibutramine in rat liver microsomes and in primary cultures of rat hepatocytes

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## Abstract

Sibutramine is an anti-obesity drug sold as a racemic mixture under the trademark Meridia or Reductil. With the aim of evaluating the stereoselectivity in phase I of sibutramine biotransformation, the formation of the main metabolites from *R*-sibutramine, *S*-sibutramine and *rac*-sibutramine was studied in rat microsomes and primary cultures of hepatocytes. A novel analytical method for the determination of sibutramine and its phase I metabolites in culture medium and microsomal incubates using isocratic reversed-phase liquid chromatography with UV detection was developed. Only two metabolites, mono-desmethylsibutramine (M1) and di-desmethylsibutramine (M2), were found in the rat microsomes incubated with sibutramine and NADPH. The kinetics of M1 and M2 formation slightly differed depending on the enantiomeric form of the sibutramine used. The stereoselectivity in sibutramine biotransformation was much more evident in primary cultures of rat hepatocytes. While *R*-sibutramine incubation led to the formation of M1 and M2 metabolites only, the incubation of *S*-sibutramine or *rac*-sibutramine (to a lesser extent) resulted in four major metabolites (M1, M2, M3 and M4) and 2 or 3 minor metabolites. On the basis of our results, *R*-sibutramine might represent the more advantageous sibutramine enantiomer from the pharmacokinetic standpoint.

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## Introduction

Nowadays, obesity represents a serious problem, especially in American and European populations. Pharmacotherapy in combination with a reduced calorie diet is recommended for obese patients as a multi-modal approach to weight loss. Sibutramine hydrochloride monohydrate represents one of the few established and well-proven agents available for treatment of obesity (Arterburn et al 2004; Ryan 2004). Sibutramine (*N*-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-*N*,*N*-dimethylamine) is a tertiary amine with one chiral centre. It is sold as a racemic mixture under the trade-name Meridia or Reductil. It acts as a monoamine-reuptake inhibitor. The weight loss of patients induced by sibutramine is thought to be due to a combination of serotonin- and noradrenaline (norepinephrine)-mediated mechanisms that increase both satiety and energy expenditure (Stock 1997; Heal et al 1998; Luque & Rey 1999).

In organisms sibutramine is rapidly demethylated to form metabolites M1 (*N*-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-*N*-methylamine) and M2 (1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutylamine). M1 and M2 undergo hydroxylation and conjugation to form inactive metabolites (Stock 1997; Hind et al 1999; Chen et al 2003). The pharmacological effects of sibutramine are mostly attributable to the M1 and M2 metabolites as these metabolites inhibit monoamine reuptake in-vitro more effectively than the parent drug (Connoley et al 1999). Sibutramine itself has several undesirable effects, the most problematic of which is increases in blood pressure and heart rate (Stock 1997). From this point of view, the M1 and M2 metabolites are considered to be safer than the parent compound (Glick et al 2000).

Sibutramine and both M1 and M2 metabolites are chiral compounds. The enantioselective pharmacodynamic profile of these enantiomers has been reported. The

*R*-enantiomers act as more potent monoamine reuptake inhibitors than the *S*-enantiomers (Glick et al 2000).

The aim of this study was to evaluate the stereoselectivity in phase I of sibutramine biotransformation in rat. The in-vitro formation of the main metabolites from *R*-sibutramine, *S*-sibutramine and *rac*-sibutramine were studied and compared. Primary cultures of rat hepatocytes and microsomal fraction of rat liver homogenates were used as model systems. A novel analytical method for determination of sibutramine and its phase I metabolites in culture medium and microsomal incubates, using isocratic reversed-phase liquid chromatography with UV detection, was developed.

## Materials and Methods

### Chemicals

Sibutramine hydrochloride, desmethylsibutramine hydrochloride (metabolite M1) and didesmethylsibutramine hydrochloride (metabolite M2) were prepared as racemic mixture using the experimental procedure according to Jeffery et al (1996) at the Department of Organic chemistry, Faculty of Pharmacy (Hradec Králové, Czech Republic). *R*- and *S*-sibutramine hydrochloride enantiomers (purity ≥ 99%) were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czech Republic). Ethyl acetate and toluene for extraction (liquid chromatography grade), acetonitrile and methanol (all of the purity grade for LC) were obtained from Merck (Darmstadt, Germany). All other chemicals were analytical grade.

### Animals

Male Wistar rats (10–12 weeks) were obtained from BioTest (Konárovice, Czech Republic). They were kept on standard rat chow with free access to tap water, in animal quarters under a 12-h light–dark cycle. The 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). The ethical committee approval no. 28999/2001-30 for the study is deposited at the Ministry of Education, Youth and Sports of the Czech Republic.

### Isolation of microsomal fraction

Livers of 6 male Wistar rats (10–12 weeks) were used as the source of microsomes. The microsomal fractions were prepared by a procedure described by Gillette (1971) with slight modification (Szotáková et al 2004). The protein content was determined by the bicinchoninic acid method (Smith et al 1985).

### Isolation of hepatocytes

Hepatocytes were obtained from the livers of 6 male rats by a two-step collagenase method (Berry et al 1991) and the isolated hepatocytes were mixed together. Three million viable (85–90%) cells in 3 mL of culture medium were placed into 60-mm plastic dishes pre-coated with collagen as described elsewhere (Szotáková et al 2004). The cultures were kept at 37°C in an atmosphere of humid air with 5% $CO_2$.

### Incubation of microsomal fraction with *R*-, *S*- and racemic sibutramine

The stock solutions (3 mM) of each substrate and NADPH were prepared by dissolving the corresponding amount of salt in re-distilled water. The other solutions of the substrates were prepared by diluting the stock solution with water. The reaction mixture consisted of 50 $\mu$L microsomal fraction (0.1 mg of proteins/mL), 100 $\mu$L NADPH (final concentration 1 mM), 100 $\mu$L substrate (final concentration 0.01–1 mM) and 50 $\mu$L 0.1 M sodium phosphate buffer, pH 7.4. The total volume of the reaction mixture was 300 $\mu$L. The standard incubation lasted 20 min. All the incubations were performed at 37°C. The reaction was stopped by alkalization and cooling in ice.

### Incubation of hepatocytes primary culture with *R*-, *S*- and racemic sibutramine

The stock solutions (3 mM of the substrates in water) were added into a fresh culture medium. The concentration of water in culture medium did not exceed 3% (v/v). Hepatocyte monolayers (18–24 h after isolation) were incubated with substrates at 37°C in an atmosphere of humid air with 5% $CO_2$. The kinetic study was performed with substrate concentrations of 10, 20, 35 and 50 $\mu$M, and the incubation lasted 4 h. In the time-dependence study, 35 $\mu$M substrate was used and 0.5-mL samples of medium were collected at intervals of 1, 2, 4 and 8 h. The medium samples were stored frozen at −80°C before their extraction.

### Cytotoxicity assay

Cytotoxicity was assayed using the MTT (dimethylthiazol diphenyl tetrazolium bromide) test as described by Denizat & Lang (1986). The cells seeded in 96-well plates were incubated with the substrate at various concentrations (5–70 $\mu$M) for 8 h. The absorbance of formazan at 595 nm in the cells treated with substrate was compared with that in control cells exposed to medium with 3% v/v water alone.

### Extraction

Before the extraction procedure, the pH of sibutramine incubates was adjusted. The best recovery of metabolites from the culture medium and microsomal incubates (approx. 80% and 94%, respectively) was obtained after alkalization of samples with sodium hydroxide (0.1 M) to pH 9.6–9.7.

All the incubates were extracted twice with double volumes of ethyl acetate. The extracts were pooled and evaporated to dryness using a rotary vacuum concentrator

Eppendorf 5301 at 30°C. The dry samples were dissolved in the mobile phase prior to the injection in HPLC apparatus.

### Chromatography conditions

The HPLC analyses of sibutramine and its metabolites, M1 and M2, were performed on an Agilent technologies 1100 series liquid chromatograph with a diode array detector. The chromatographic separation was performed using a Zorbax Eclipse XDB-C8 column, $4.6 \times 150$ mm with 5 $\mu$m particles (Agilent Technologies, USA). The mobile phase consisted of ammonium phosphate buffer (20 mM, pH 6.0 adjusted with phosphoric acid)–acetonitrile–methanol (42:22:36 v/v/v), the flow rate was 1.5 mL min$^{-1}$, temperature 25°C and detection wavelength 223 nm.

### Identification of metabolites of sibutramine

The metabolites M1 and M2 were collected separately into glass test tubes after chromatographic separation using the conditions described above. After basification with sodium hydroxide, the analytes were extracted twice with double volumes of toluene. The extracts were pooled and evaporated to dryness. The residue was dissolved in a mixture of water–methanol (2:3 v/v) and analysed using an LCQ Advantage mass spectrometer (ThermoFinnigan, USA). The samples were injected into the ion source (ESI) using a 250-$\mu$L syringe and positive ion mass spectra, MS and MS/MS, were recorded.

### Statistical analysis

The differences in the biological properties of the individual compounds were statistically determined using a one-way analysis of variance in conjunction with a Dunnett's post-hoc test.

## Results

### HPLC method

An analytical column with high pure silica gel and end-capped stationary phase Zorbax Eclipse XDB-C8 was chosen to avoid the peak-tailing problem due to the basic character of sibutramine and its metabolites. It was found that the resolution of the metabolites strongly depended on the pH of the buffer and an accuracy of pH $6.00 \pm 0.05$ was necessary. The retention time of sibutramine, M1, M2, M3 and M4 was 31.0, 7.7, 9.1, 4.4 and 6.1 min, respectively.

### Incubation of microsomal fraction with *R*-, *S*- and *rac*-sibutramine

The rat hepatic microsomes were incubated with substrate (*rac*-sibutramine, *R*-sibutramine or *S*-sibutramine) and coenzyme NADPH. The metabolites M1 and M2 were found to be the only biotransformation products of sibutramine in rat microsomes. The kinetics of M1 and M2 formation was studied within the substrate concentration range of 0.01–1.0 mM. A blank sample was analysed simultaneously and no peak interfering with sibutramine and its metabolites was found.

Figure 1A demonstrates the plots of rate of M1 formation vs substrate concentration. Detailed analysis showed that the data are described better by a sigmoidal dose–response curve rather than by the Michaelis–Menten equation. The apparent kinetic parameters for microsomal M1 formation and intrinsic clearance were calculated

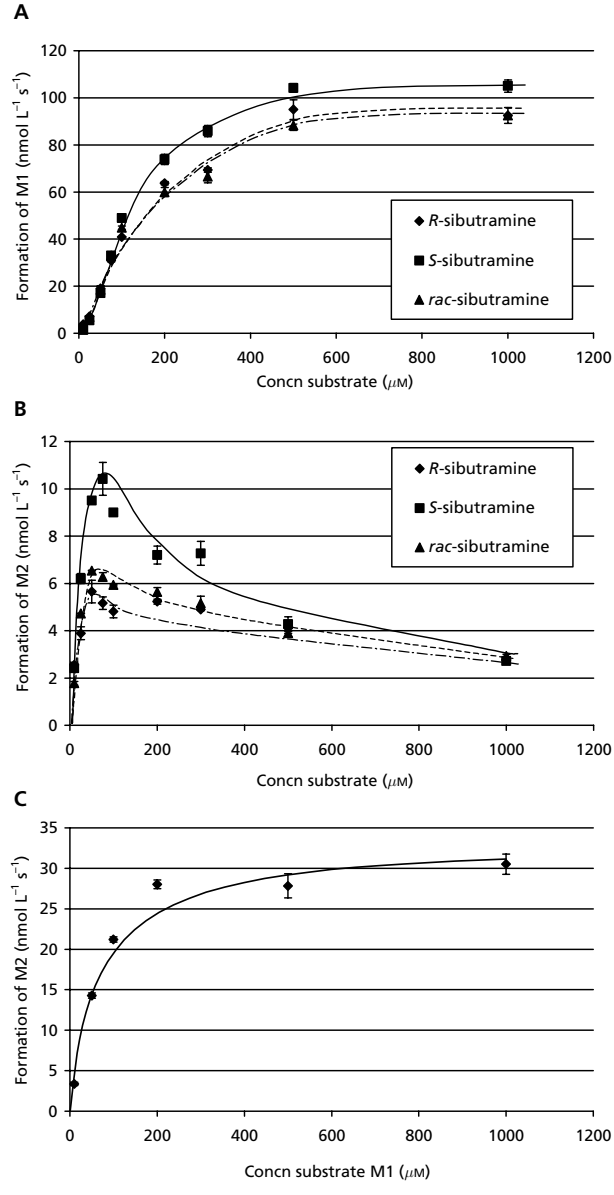


**Figure 1** Velocity of formation of metabolites, depending on substrate concentration, in rat microsomes. Formation of M1 from *R*-, *S*- or *rac*-sibutramine (A), formation of M2 from *R*-, *S*- or *rac*-sibutramine (B), formation of M2 from M1 (C).

**Table 1** Apparent kinetic parameters for M1 and M2 formation from *R*-, *S*-, *rac*-sibutramine and for M2 formation from *rac*-M1 in rat hepatic microsomes

| Substrate | $K_m'$ ± s.d. ($\mu$mol $L^{-1}$) | | $V_{max}'$ ± s.d. ($\mu$mol $L^{-1}$ $s^{-1}$) | | $Cl_{int}$ ($10^{-4}$ $s^{-1}$) | |
|---|---|---|---|---|---|---|
| | M1 | M2 | M1 | M2 | M1 | M2 |
| *R*-Sibutramine | 173.1 ± 35.48 | 25.5 ± 9.7 | 0.118 ± 0.009* | 0.008 ± 0.001* | 6.8 | 3.1 |
| *S*-Sibutramine | 171.0 ± 45.08 | 49.6 ± 17.0 | 0.134 ± 0.003* | 0.018 ± 0.003* | 7.8 | 3.7 |
| *rac*-Sibutramine | 167.1 ± 28.07 | 54.9 ± 25.0 | 0.114 ± 0.007 | 0.014 ± 0.004 | 6.8 | 2.6 |
| *rac*-M1 | — | 57.6 ± 10.5 | — | 0.033 ± 0.001 | — | 5.7 |

Each value represents the mean ± s.d. of three independent experiments (n = 3). The microsomes were obtained from livers of six rats. *$P \leq 0.05$ in *R*- vs *S*-enantiomer.

using software GraphPad Prism version 3.00 (1999; GraphPad Software Inc., San Diego CA); the data are presented in Table 1. No significant differences in $K_m'$ between sibutramine enantiomers were observed. On the other hand, microsomal enzymes displayed slightly, but significantly ($P < 0.05$), higher $V_{max}'$ for *S*-sibutramine as compared with *R*- or *rac*-sibutramine.

The plots of the rate of M2 formation vs sibutramine concentration are shown in Figure 1B. The curves obtained were non-hyperbolic. At low sibutramine concentration, the velocity of M2 formation linearly increased with increasing concentration of sibutramine to reach the maximum, and then it decreased with increasing sibutramine concentration. When M1 was used as the substrate instead of sibutramine, the plots of M2 formation approached the classical hyperbolic curve (Figure 1C). The apparent kinetic parameters for microsomal M2 formation calculated are presented in Table 1. Microsomal enzymes displayed significantly higher values of $K_m'$ and $V_{max}'$ for *S*-sibutramine than for *R*-sibutramine. When M1 was used as the substrate instead of sibutramine, a significantly higher value of $V_{max}'$ was found. Intrinsic clearance was not affected by enantiomeric form of sibutramine.

### Incubation of hepatocytes with *R*-, *S*- and racemic sibutramine

Before the biotransformation study, the cytotoxicity of sibutramine in primary cultures of rat hepatocytes was assayed using the MTT test. No significant decrease in the viability of cells caused by *R*-, *S*- or *rac*-sibutramine was observed up to a concentration of 50 $\mu$M.

The time and concentration dependence of phase I biotransformation of *R*-, *S*- and *rac*-sibutramine was studied in hepatocytes. When *R*-sibutramine was used as the substrate, M1 and M2 represented the only main metabolites in the culture medium. In addition to M1 and M2, two other major metabolites (denoted as M3 and M4) and 2 or 3 minor metabolites were found as biotransformation products of *S*- and *rac*-sibutramine. By means of mass spectroscopy, M3 ($Mr = 267\ g\ mol^{-1}$) was identified as a hydroxy derivative of M2. The precise position of the OH-group in M3 is not yet known, and also the structure of M4 ($Mr = 309\ g\ mol^{-1}$) is under study. M1 and M2 were quantified using calibration curves of standards. The area under the peak was used for semi-quantification of the metabolites M3 and M4 as no standards were available.

The time dependence of formation of metabolites M1, M2, M3 and M4 is presented in Figure 2. The concentration of active metabolites M1 and M2 increased with incubation time and culminated in 2 and 4 h, respectively. A longer time of incubation led to lowering of the concentrations of these metabolites in the medium. When *S*-sibutramine was used as the substrate, the concentration of active metabolites M1 and M2 after 8 h incubation was significantly lower than when *R*- and *rac*-sibutramine were used. While the production of M4 metabolite also culminated in 2 h, the concentration of M3 increased until the end of the incubation (8 h).

The concentration dependence of formation of metabolites was tested using a concentration range of substrates 0–50 $\mu$M. No significant differences in M1 and M2 kinetics between the enantiomeric forms of sibutramine used were observed (data not shown). The results of the kinetic study of M3 and M4 formation are presented in Figure 3. Both metabolites were formed only when *S*-sibutramine or *rac*-sibutramine were used as the substrate. While the curves obtained for M3 metabolite corresponded to Michaelis–Menten kinetics (Figure 3A), those obtained for M4 metabolite corresponded to the sigmoidal dose–response kinetics (Figure 3B), which can be explained either by the dimeric form of the respective native biotransformation enzyme or by the fact that M4 is formed by participation of more than one biotransformation enzyme.

## Discussion

Sibutramine is a chiral drug administered as the racemate. When the effectiveness of the enantiomers of sibutramine and its main metabolites was compared, the stereoselectivity in biological activity was reported (Glick et al 2000). This study of the biotransformation of sibutramine enantiomers was initiated with the aim of evaluating the stereoselectivity in sibutramine metabolism. Primary cultures of rat hepatocytes and rat liver microsomes were used as model systems. At the beginning of the study, it was necessary to develop appropriate analytical methods for

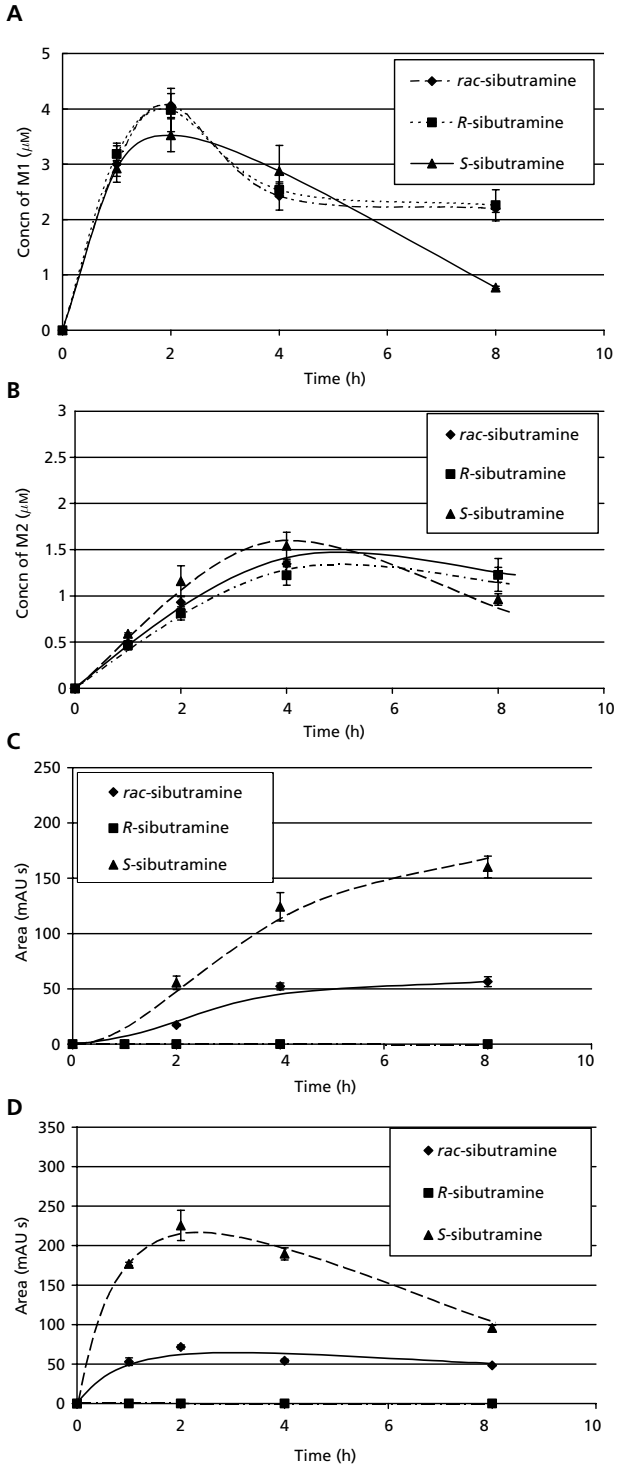


**Figure 2** Time dependence of formation of M1 (A), M2 (B), M3 (C) and M4 (D) from *R*-, *S*- or *rac*-sibutramine in primary cultures of rat hepatocytes.

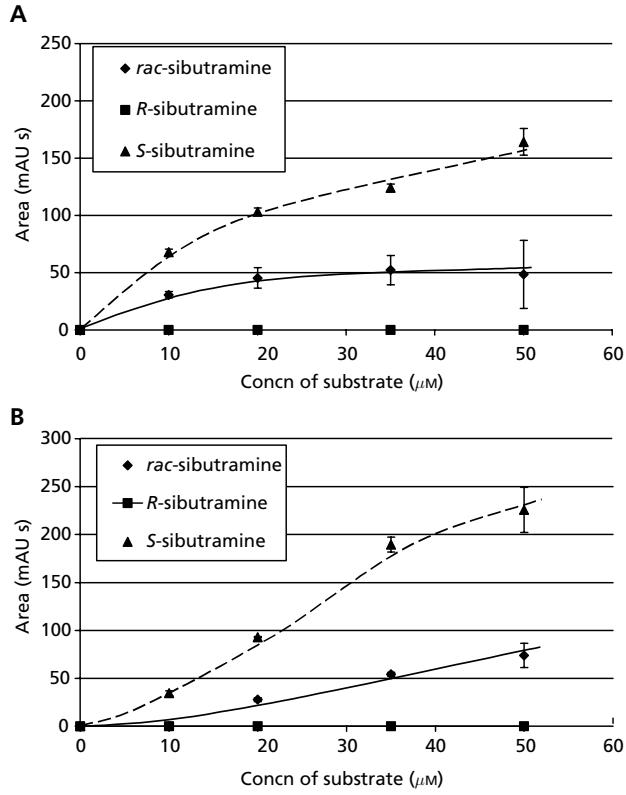


**Figure 3** Concentration dependence of formation of M3 (A) and M4 (B) from *R*-, *S*- or *rac*-sibutramine in primary cultures of rat hepatocytes.

separation and quantification of sibutramine and its metabolites. The methods previously reported (Radhakrishna et al 2000; Chen et al 2003; Ding et al 2003) were not suitable for an in-vitro biotransformation study. The novel method described herein allows the separation and quantification of sibutramine and its phase I metabolites in culture medium and microsomal incubates.

Only two metabolites, M1 (desmethylated sibutramine) and M2 (didesmethylated sibutramine), were detected in incubates of sibutramine with microsomes and NADPH regardless of the enantiomeric form of substrate used. Sibutramine acted as an inhibitor of the enzyme, which catalysed M2 formation from M1. On the other hand, pronounced stereoselectivity of sibutramine biotransformation was found in primary cultures of rat hepatocytes. While *R*-sibutramine incubation led to formation of two main metabolites (M1 and M2) only, incubation of *S*-sibutramine or *rac*-sibutramine (to a lesser extent) resulted in four major metabolites (M1, M2, M3 and M4) and 2 or 3 minor metabolites. On the basis of these results, *R*-sibutramine could be considered as being the enantiomer with less extensive biotransformation than the *S*-enantiomer by rat hepatocytes in-vitro. Lower biotransformation might mean slower deactivation and a lower risk of drug–drug interactions and inter-individual variability. Moreover, both *R*-desmethylsibutramine and *R*-didesmethylsibutramine were clearly more potent in depressing food intake and decreasing body weight than the *S*-enantiomers (Glick et al 2000). Thus, *R*-sibutramine might represent the more advantageous sibutramine enantiomer from a pharmacokinetic as well as a pharmacodynamic point of view. The results

obtained in rat should be considered as preliminary with respect to well-known inter-species differences in activity, stereoselectivity and stereospecificity of biotransformation enzymes. Our results presented here seem to be so promising that a biotransformation study of sibutramine using human samples should be initiated.

## Conclusion

This biotransformation study of the new anti-obesity drug sibutramine in rat liver microsomes and in primary cultures of rat hepatocytes indicates that the phase I metabolism of sibutramine is stereoselective. In hepatocytes, *R*-sibutramine incubation led to formation of M1 and M2 metabolites only (as well as in microsomes); incubation of *S*-sibutramine resulted in four major metabolites (M1, M2, M3 and M4). Lower biotransformation of *R*-sibutramine, together with its higher effectiveness in depressing food intake and decreasing body weight (Glick et al 2000), could make this enantiomer more advantageous.

## References


Arterburn, D. E., Crane, P. K., Veenstra, D. L. (2004) The efficacy and safety of sibutramine for weight loss. A systematic review. *Arch. Intern. Med.* **9**: 994–1003

Berry, M. N., Edwards, A. M., Barritt, G. J. (1991) Isolated hepatocytes – preparation, properties and applications. In: van der Vliet, P. C., Pillai, S. (eds) *Laboratory techniques in biochemistry and molecular biology*. Elsevier Science, Amsterdam, pp 1–470

Chen, J., Lu, W., Zhang, Q., Jiang, X. (2003) Determination of the active metabolite of sibutramine by liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B* **785**: 197–203

Connoley, I. P., Liu, Y. L., Frost, I., Reckless, I., Heal, D. J., Stock, M. J. (1999) Thermogenic effects of sibutramine and its metabolites. *Br. J. Pharmacol.* **126**: 1487–1495

Denizat, F., Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. *J. Immunol. Methods* **89**: 271–277

Ding, L., Hao, X., Huang, X., Zhang, S. (2003) Simultaneous determination of sibutramine and its N-desmethyl metabolites in human plasma by liquid chromatography-electrospray ionization-mass spectrometry. Methods and clinical application. *Anal. Chim. Acta* **492**: 241–248

Gillette, J. R. (1971) Techniques for studying drug metabolism in vitro. In: La Du, B. N., Mandel, H. G., Way, E. L. (eds) *Fundamentals of drug metabolism and drug disposition*. The Williams and Wilkins Company, Baltimore, pp 400–418

Glick, S. D., Haskew, R. E., Maisonneuve, M. (2000) Enantioselective behavioral effects of sibutramine metabolites. *Eur. J. Pharmacol.* **397**: 93–102

Heal, D. J., Aspley, S., Prow, M. R., Jackson, H. C., Martin, K. F., Cheetham, S. C. (1998) Sibutramine: a novel anti-obesity drug. A review of the pharmacological evidence to differentiate it from D-amphetamine and D-fenfluramine. *Int. J. Obesity* **22**: S18–S28

Hind, I. D., Mangham, J. E., Ghani, S. P. (1999) Sibutramine pharmacokinetics in young and elderly healthy subjects. *Eur. J. Clin. Pharmacol.* **54**: 847–849

Jeffery, J. E., Kerrigan, F., Miller, T. K., Smith, G. J., Tometzki, G. B. (1996) Synthesis of sibutramine, a novel cyclobutylalkylamine useful in the treatment of obesity, and its major human metabolites. *J. Chem. Soc. Perkin Trans.* **1**: 2583–2590

Luque, C. A., Rey, J. A. (1999) Sibutramine: a serotonin-norepinephrine reuptake inhibitor for the treatment of obesity. *Ann. Pharmacother.* **33**: 968–978

Radhakrishna, T., Narayana, C. L., Rao, D. S., Vyas, K., Reddy, G. O. (2000) LC method for determination of assay and purity of sibutramine hydrochloride and its enantiomers by chiral chromatography. *J. Pharm. Biomed. Anal.* **22**: 627–639

Ryan, D. H. (2004) Clinical use of sibutramine. *Drugs Today* **40**: 41–54

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, M. D., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**: 76–85

Stock, M. J. (1997) Sibutramine: a review of the pharmacology of a novel anti-obesity agent. *Int. J. Obesity* **21**: S25–S29

Szotáková, B., Skálová, L., Baliharová, V., Dvorščáková, M., Štorkánová, L., Šišpera, L., Wsól, V. (2004) Characterization of enzymes responsible for biotransformation of the new anti-leukotrienic drug quinlukast in rat liver microsomes and in primary cultures of rat hepatocytes. *J. Pharm. Pharmacol.* **56**: 205–212