Pergamon

0960-0760(95)00144-1

J. Steroid Biochem. Molec. Biol. Vol. 54, No. 5/6, pp. 281–285, 1995

0960-0760/95 $9.50 + 0.00

Inhibition of Adrenal Steroid Metabolism by Administration of 1-Aminobenzotriazole to Guinea Pigs

Dan Xu,[1] Jeffrey M. Voigt,[1] Bruce A. Mico[2] and Howard D. Colby[1]*

[1]Department of Pharmacology and Toxicology, Philadelphia College of Pharmacy and Science, 600 South 43rd Street, Philadelphia, PA 19104, U.S.A. and [2]Department of Drug Metabolism and Pharmacokinetics, Hoffman-LaRoche, Inc., 340 Kingsland Street, Nutley, NJ 07110, U.S.A.

Prior *in vitro* investigations demonstrated that the *P*450 suicide substrate, 1-aminobenzotriazole (ABT), was a potent inhibitor of xenobiotic metabolism but had no effect on steroidogenic enzymes in the guinea pig adrenal cortex. Studies were done to determine if ABT administration to guinea pigs *in vivo* also selectively inhibited adrenal xenobiotic metabolism. At single doses of 25 or 50 mg/kg, ABT effected rapid decreases in spectrally detectable adrenal *P*450 concentrations. The higher dose caused approx. 75% decreases in microsomal and mitochondrial *P*450 levels within 2 h. The decreases in *P*450 were sustained for 24 h but concentrations returned to control levels within 72 h. Accompanying the ABT-induced decreases in adrenal *P*450 content were proportionately similar decreases in *P*450-mediated xenobiotic and steroid metabolism. Microsomal benzo(a)pyrene hydroxylase, benzphetamine *N*-demethylase, 17α-hydroxylase and 21-hydroxylase activities were decreased to 20–25% of control values by the higher dose of ABT. Mitochondrial 11β-hydroxylase and cholesterol sidechain cleavage activities were similarly diminished by ABT treatment. Adrenal 3β-hydroxysteroid dehydrogenase activity, by contrast, was not affected by ABT, indicating specificity for *P*450-catalyzed reactions. The results demonstrate that ABT *in vivo* is a non-selective inhibitor of adrenal steroid- and xenobiotic-metabolizing *P*450 isozymes. The absence of ABT effects on steroid metabolism *in vitro* suggests that an extra-adrenal metabolite may mediate the *in vivo* inhibition of steroidogenesis.

J. Steroid Biochem. Molec. Biol., Vol. 54, No. 5/6, pp. 281–285, 1995

INTRODUCTION

The cytochrome *P*450 isozymes that are expressed in the adrenal cortex are principally involved in the biosynthesis of steroid hormones [1, 2]. However, in several species adrenal metabolism of foreign compounds has also been demonstrated [3–5]. Most of the xenobiotic-metabolizing activity in adrenal glands has been attributed to different *P*450 isozymes than those involved in steroidogenesis [6, 7], but relatively little is known about the enzymes involved.

In the guinea pig adrenal cortex, the metabolism of foreign compounds is highly correlated with the presence of a microsomal *P*450 having an apparent mol. wt. of 52 kDa [6]. The concentration of this isozyme and xenobiotic-metabolizing activities are far greater in the zona reticularis (ZR) than in the outer zones of the gland, and both are suppressed by ACTH treatment [6, 8]. Regulation of this 52 kDa isozyme, in general, seems to be independent of the microsomal steroid hydroxylases, *P*450c17 and *P*450c21 [3, 6, 8]. However, since this isozyme has yet to be purified or cloned, detailed information on its catalytic capabilities and regulatory mechanisms are not yet available.

We recently reported that incubation of guinea pig adrenal microsomal preparations with 1-aminobenzotriazole (ABT), a mechanism-based (suicide) *P*450 inhibitor [9, 10], caused almost complete loss of xenobiotic-metabolizing activity without any effect on steroid hydroxylation reactions [11]. As expected for a *P*450 suicide substrate, enzyme inactivation by ABT was both NADPH- and time-dependent. These results suggested that ABT might serve as a useful probe for further studies on the *P*450 isozyme(s) involved in adrenal xenobiotic metabolism. However, for greatest

*Correspondence to H. D. Colby.
Received 30 Jan. 1995; accepted 13 Apr. 1995.

usefulness, the selectivity of ABT for xenobiotic metabolism should be applicable *in vivo* as well as *in vitro*. Accordingly, the studies presented in this report were done to investigate the effects of ABT administration to guinea pigs on adrenal xenobiotic and steroid metabolism.

MATERIALS AND METHODS

ABT was supplied by Hoffman-LaRoche, Inc., Nutley, NJ. A polyclonal antibody to *P*4501A1 and *P*4501A2 that cross-reacts with a 52 kDa isozyme in guinea pig adrenal microsomes was obtained from Human Biologics, Inc. Phoenix, AZ. The 3β-hydroxysteroid dehydrogenase inhibitor, 4,4-dimethyl-2α-cyano-20-spirox-5-en-3-one, was provided by Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. Organic solvents of the highest grades commercially available were obtained from Fisher Scientific, Pittsburgh, PA. Other reagents were obtained from Sigma Chemical Co., St Louis, MO.

Male English Short-Hair guinea pigs weighing approx. 800–1000 g were obtained from Camm Research Institute, Wayne, NJ. Animals were maintained under standardized conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°C) and received food and water *ad libitum*. For most investigations, ABT was administered between 4:00 and 5:00 p.m. as a single i.p. injection at a dose of 25 or 50 mg/kg body wt. in 0.9% saline; controls received the vehicle only. Guinea pigs were killed the following day between 8:00 and 9:00 a.m. by CO_2 inhalation. For the time-course study (Table 1), ABT was given between 8:00 and 9:00 a.m. and animals were killed 1, 2, 4, 24, 48 or 72 h later. At the time of killing, adrenal glands were quickly removed and placed in cold 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4) on ice. Tissues were then trimmed free of fat and connective tissue, weighed, and homogenized in sucrose–Tris buffer. Washed mitochondrial and microsomal fractions were prepared by differential centrifugation [12, 13] and used for the assays described below.

Adrenal mitochondrial 11β-hydroxylase activity was assayed as the rate of conversion of 11-deoxycortisol to cortisol as described previously [12]. Cortisol was measured fluorometrically [14]. Cholesterol sidechain cleavage activity was determined as the rate of pregnenolone production by isolated adrenal mitochondria, with endogenous cholesterol as the substrate [12]. Cholesterol metabolism was initiated by the addition of 10 mM sodium isocitrate, and 4,4-dimethyl-2α-cyano-20-spirox-5-en-3-one (50 μM), a 3β-hydroxysteroid dehydrogenase inhibitor, was included in each flask to prevent the conversion of pregnenolone to progesterone [15]. Pregnenolone was extracted from the incubation flasks with methylene dichloride and measured with a highly specific radioimmunoassay [12].

Microsomal steroid 21-hydroxylase activity was determined from the rate of conversion of 17α-hydroxyprogesterone to 11-deoxycortisol, and 17α-hydroxylase activity from the rate of conversion of progesterone to 17α-hydroxyprogesterone plus 11-deoxycortisol. Incubation conditions and HPLC analyses of metabolites were previously described in detail [16]. 3β-Hydroxysteroid dehydrogenase-isomerase activity in adrenal microsomes was measured as the rate of conversion of pregnenolone to progesterone utilizing HPLC for metabolite separation and UV absorbance at 254 nm for quantitation [16]. Benzo[a]pyrene hydroxylation was determined by the fluorometric method of Nebert and Gelboin [17]. Quinine sulfate was calibrated against authentic 3-hydroxy-benzo[a]pyrene and routinely used as the fluorescence standard. Benzphetamine N-demethylation was assayed as the amount of formaldehyde formed using the method of Nash [18], as previously described [19]. For all enzyme assays, conditions were established to ensure linearity of product formation with respect to protein concentrations and incubation times.

Cytochrome *P*450 was measured as the dithionite-reduced CO complex as described by Omura and Sato [20]. Substrate-induced type I difference spectra were recorded with an Aminco DW-2a recording spectrophotometer in the split-beam mode. For Western blot analyses, the anti-*P*450c17 and anti-*P*450c21 antisera were prepared as described previously [21]. These antisera recognize adrenal microsomal proteins having mol. wt. of 54 and 50 kDa, respectively. A polyclonal antibody to *P*4501A1 and *P*4501A2 was used to monitor the 52 kDa *P*450 isozyme in adrenal microsomes [3, 4]. This antibody reacts with the microsomal protein having a mol. wt. of 52 kDa but not with the 54 kDa (*P*450c17) or 50 kDa (*P*450c21) protein. Similar observations have been made by Black *et al.* [3, 4] using a different polyclonal antibody raised against *P*4501A1 and *P*4501A2. SDS–PAGE was done by the method of Laemmli [22] and Western blots of microsomal proteins by the method of Towbin *et al.* [23]. Immunoreactive proteins were visualized by peroxidase immunostaining. Microsomal and mitochondrial protein concentrations were determined by the method of Lowry *et al.* [24]. Statistical analyses of differences between group means were done with the Students *t*-test or Newman–Keuls multiple-range test. Data are presented as means ± SEM.

RESULTS AND DISCUSSION

Administration of ABT to guinea pigs at a dose of 50 mg/kg body wt. caused rapid decreases in adrenal mitochondrial and microsomal cytochrome *P*450 concentrations (Table 1). Within 1 h after an i.p. injection of ABT, *P*450 levels in both subcellular fractions decreased significantly. The maximal declines (70–80%) occurred within approx. 2 h and

*Table 1. Adrenal cytochrome P450 concentrations at various times after ABT administration to guinea pigs**

	Controls	ABT-treated 1 h	2 h	4 h	24 h	48 h	72 h
Mitochondria							
*P*450 (nmol/mg protein)	1.0 ± 0.2	0.6 ± 0.1†	0.3 ± 0.1†	0.4 ± 0.1†	0.3 ± 0.1†	0.6 ± 0.1†	1.1 ± 0.2
Microsomes							
*P*450 (nmol/mg protein)	1.8 ± 0.2	1.0 ± 0.2†	0.4 ± 0.1†	0.3 ± 0.1†	0.4 ± 0.1†	0.9 ± 0.2†	1.6 ± 0.3

*ABT was administered at a dose of 50 mg/kg and *P*450 assays were done as described in Methods. Values are expressed as means ± SEM of 4–5 animals per group. Because control values were similar at all time points, they were combined.
†$P < 0.05$ (vs control value).

were sustained for at least 24 h. After 48 h, some recovery of *P*450 levels was observed, and within 72 h after ABT administration, mitochondrial and microsomal *P*450 concentrations returned to control values (Table 1).

We previously found that ABT *in vitro* had no effect on mitochondrial *P*450 concentrations and decreased microsomal levels maximally by about 35% [11]. The far greater losses of *P*450 that occurred after ABT treatment *in vivo* suggested that ABT had less selectivity *in vivo* than *in vitro*. This hypothesis was confirmed by determination of adrenal xenobiotic- and steroid-metabolizing activities. As shown in Fig. 1, ABT administration at doses of 25 or 50 mg/kg body wt. caused marked decreases in microsomal benzo(a)pyrene (BP) hydroxylase and benzphetamine (BZ) *N*-demethylase activities, effects like those produced *in vitro* [11]. However, in contrast to its selectivity for xenobiotic metabolism *in vitro*, ABT administration *in vivo* also decreased steroid hydroxylase activities (Fig. 2). Microsomal 17α-hydroxylase and 21-hydroxylase activities were decreased to a proportionately similar extent as xenobiotic metabolism. The non-selectivity of ABT actions *in vivo* extended to mitochondrial as well as microsomal *P*450-mediated steroid metabolism. ABT administration decreased both 11β-hydroxylase and cholesterol sidechain cleavage activities in adrenal mitochondria (Fig. 3). Neither of these reactions was affected by mitochondrial incubations with ABT *in vitro* [11]. ABT treatment had no effect on microsomal 3β-hydroxysteroid dehydrogenase-isomerase activity (data not shown), a non-*P*450 dependent reaction involved in steroid hormone biosynthesis [1, 2]. The latter suggests some degree of specificity for *P*450-catalyzed reactions by ABT.

The effects of ABT treatment on the binding of steroid and xenobiotic substrates to microsomal *P*450 isozymes, as indicated by type I spectral changes (Fig. 4), were also indicative of non-selectivity *in vivo*. At both doses administered, ABT caused quantitatively similar declines in the magnitudes of the BZ- and progesterone-induced spectra, demonstrating equipotent effects on the binding of both substrates to their respective *P*450 isozymes. PAGE and Western blot analyses revealed that ABT treatment had no apparent effect (data not shown) on microsomal concentrations of the apoproteins corresponding to *P*450c17, *P*450c21, or the 52 kDa isozyme highly correlated with xenobiotic metabolism [6, 8]. This is not necessarily surprising since it is the prosthetic heme group of cytochrome *P*450 that is alkylated by the ABT

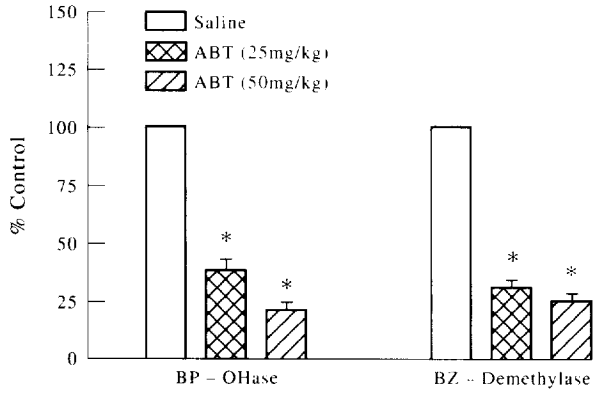

Fig. 1. Effects of ABT administration on adrenal microsomal benzo(a)pyrene hydroxylase (BP-OHase) and benzphetamine (BZ) demethylase activities. Animals were pretreated for approx. 16 h and enzyme activities determined as described in Methods. Data are expressed as percent of control values and are the means ± SEM of 5–6 animals in each group. 100% equivalent to: 435 ± 38 pmol/min/mg protein, BP-OHase; 8.6 ± 1.2 nmol/min/mg protein, BZ-demethylase. *$P < 0.05$ (vs controls).

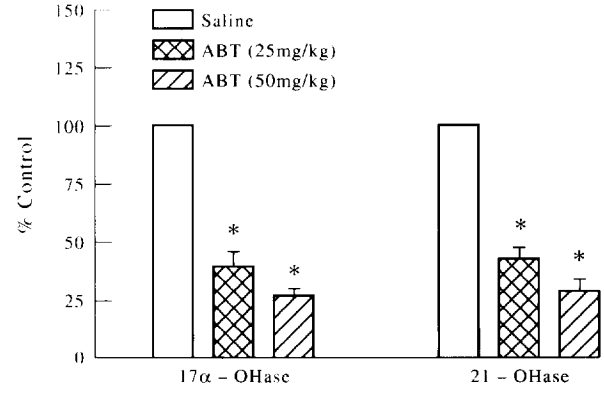

Fig. 2. Effects of ABT administration on adrenal microsomal 17α-hydroxylase (17α-OHase) and 21-hydroxylase (21-OHase) activities. Animals were pretreated for approx. 16 h and enzyme activities determined as described in Methods. Data are expressed as percent of control values and are the means ± SEM of 5–6 animals in each group. 100% equivalent to: 9.4 ± 0.8 nmol/min/mg protein, 17α-OHase; 3.9 ± 0.5 nmol/min/mg protein, 21-OHase. *$P < 0.05$ (vs controls).

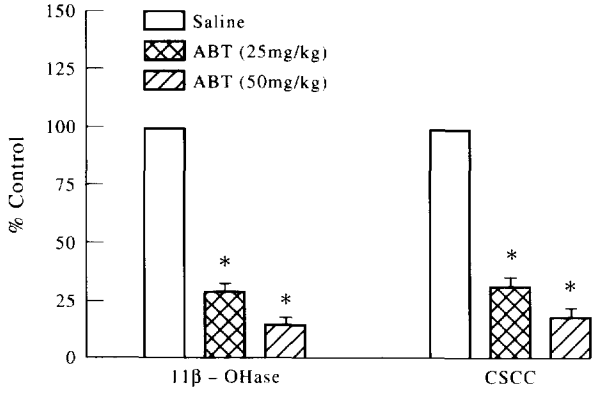

Fig. 3. Effects of ABT administration on adrenal mitochondrial 11β-hydroxylase (11β-OHase) and cholesterol sidechain cleavage (CSCC) activities. Animals were pretreated for approx. 16 h and enzyme activities determined as described in Methods. Data are expressed as percent of control values and are the means ± SEM of 5–6 animals in each group. 100% equivalent to: 1.6 ± 0.3 nmol/min/mg protein, 11β-OHase; 0.17 ± 0.03 nmol/min/mg protein, CSCC. *$P < 0.05$ (vs controls).

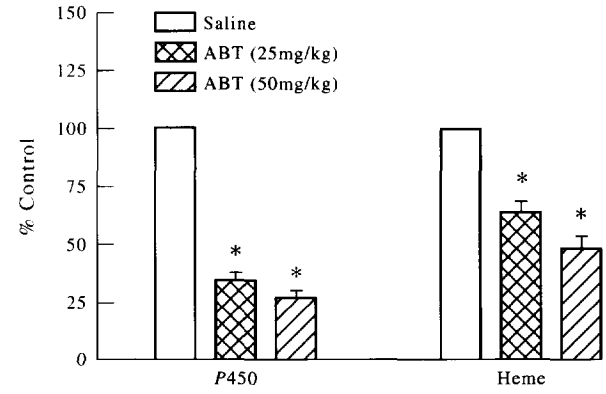

Fig. 5. Effects of ABT administration on adrenal microsomal cytochrome P450 and heme concentrations. Animals were pretreated for approx. 16 h and assays done as described in Methods. Data are expressed as percent of control values and are the means ± SEM of 5–6 animals in each group. 100% equivalent to 1.8 ± 0.1 nmol/mg protein, P450; 3.7 ± 0.3 nmol/mg protein, heme. *$P < 0.05$ (vs controls).

derivative, benzyne, causing loss of activity [9, 10]. However, we cannot exclude the possibility that ABT caused some modification of apoproteins, but without loss of immunoreactivity. ABT administration did cause quantitatively similar decreases in heme and in spectrally detectable *P*450 concentrations in microsomes (Fig. 5) and mitochondria (not shown). We previously obtained similar results *in vitro* after incubation of adrenal microsomes with ABT [11].

The results presented in this report indicate that ABT is a potent *in vivo* inhibitor of both steroid and xenobiotic metabolism in the adrenal cortex. These observations may offer an explanation for the findings of Meschter *et al.* who reported an increase in adrenal weight [25] but decrease in plasma corticosterone concentrations (personal communication) in ABT-treated rats. It is possible that inactivation of steroidogenic *P*450 isozymes by ABT leads to a decline in adrenal corticosteroid synthesis and elicits a compensatory increase in pituitary ACTH secretion. The elevated levels of ACTH could account for the adrenal enlargement noted by Meschter *et al.* [25] but would not necessarily restore corticosterone secretion to normal because of the enzyme inactivation caused by ABT.

Further studies are now needed to determine the mechanism of action of ABT on adrenal steroid hydroxylases. In other organs it has been clearly established that ABT is a suicide substrate for various *P*450 isozymes [9, 10, 26–29]. Our prior *in vitro* investigations [11] suggest that the same mechanism is applicable to ABT inactivation of adrenal xenobiotic-metabolizing enzymes. However, the absence of effects on steroid hydroxylases *in vitro* under the same conditions that cause almost complete loss of xenobiotic metabolism [11], suggests that a mechanism other than suicide inhibition may account for the *in vivo* actions of ABT on adrenal steroid metabolism. It is also possible that an extra-adrenal metabolite of ABT, and not the parent compound, is responsible for the inactivation of steroidogenic *P*450 isozymes. Investigations to consider these as well as other possible mechanisms are currently in progress.

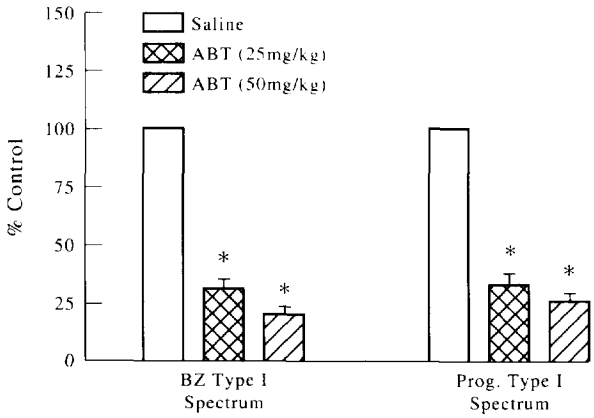

Fig. 4. Effects of ABT administration on the adrenal microsomal benzphetamine (BZ)- and progesterone (Prog.)-induced type I spectra. Animals were pretreated for approx. 16 h and assays done as described in Methods. Data are expressed as means ± SEM of 5–6 animals in each group. 100% equivalent to: 0.08 ± 0.02 ($\Delta A_{385-420}$), BZ Type I Spectrum; 0.26 ± 0.04 ($\Delta A_{385-420}$), Prog. Type I Spectrum. *$P < 0.05$ (vs controls).

Acknowledgement—These investigations were supported by Hoffman-LaRoche, Inc. and by USPHS AG-11987.

REFERENCES

1. Hall P. F.: Cytochromes *P*450 and the regulation of steroid synthesis. *Steroids* 48 (1986) 131–196.
2. Takemori S. and Kominami S.: Adrenal microsomal cytochrome *P*-450 dependent reactions in steroidogenesis and biochemical properties of the enzymes involved therein. In *Molecular Mechanisms of Adrenal Steroidogenesis and Aspects of Regulation and Application: Frontiers in Biotransformation, Volume 3* (Edited by K. Ruckpaul and H. Rein). Akademie-Verlag, Berlin (1990) pp. 153–203.

3. Colby H. D. and Rumbaugh R. C.: Adrenal drug metabolism. In *Extrahepatic Metabolism of Drugs and Other Compounds* (Edited by T. E. Gram). Spectrum Publications, NY (1980) 239–266.
4. Colby H. D. and Eacho P. I.: Chemical-induced adrenal injury: role of metabolic activation. In *Endocrine Toxicology* (Edited by J. A. Thomas, K. S. Korach and J. A. McLachlan). Raven Press, NY (1985) pp. 35–66.
5. Hallberg E.: Metabolism and toxicity of xenobiotics in the adrenal cortex, with particular reference to 7,12-dimethylbenz(a)anthracene. *J. Biochem. Toxic.* 5 (1990) 71–90.
6. Black V. H., Barilla J. R., Russo J. J. and Martin K. O.: A cytochrome *P*-450 immunochemically related to *P*-450 c, d (*P*-450I) localized to the smooth microsomes and inner zone of the guinea pig adrenal. *Endocrinology* **124** (1989) 2480–2493.
7. Otto S., Marcus C., Pidgeon C. and Jefcoate C.: A novel adrenocorticotropin-inducible cytochrome *P*-450 from rat adrenal microsomes catalyzes polycyclic aromatic hydrocarbon metabolism. *Endocrinology* **129** (1991) 970–982.
8. Black V. H.: Immunodetectable cytochromes *P*450I, II, and III in guinea pig adrenal-hormone responsiveness and relationship to capacity for xenobiotic metabolism. *Endocrinology* **127** (1990) 1153–1159.
9. Ortiz de Montellano P. R. and Mathews J. M.: Autocatalytic alkylation of the cytochrome *P*-450 prosthetic haem group by 1-aminobenzotriazole. *Biochem. J.* **195** (1981) 761–764.
10. Mico B. A., Federowicz D. A., Ripple M. G. and Kerns W.: *In vivo* inhibition of oxidative drug metabolism by, and acute toxicity of, 1-aminobenzotriazole (ABT): a tool for biochemical toxicology. *Biochem. Pharmac.* **37** (1988) 2515–2519.
11. Xu D., Voigt J. M., Mico B. A., Kominami S., Takemori S. and Colby H. D.: Inhibition of adrenal cytochromes *P*450 by 1-aminobenzotriazole *in vitro*: selectivity for xenobiotic metabolism. *Biochem. Pharmac.* **48** (1994) 1421–1426.
12. Colby H. D. and Eacho P. I.: Mitochondrial steroid metabolism in the inner and outer zones of the guinea pig adrenal cortex. *J. Steroid Biochem.* **23** (1985) 477–484.
13. Sherry J. H., O'Donnell J. P., Flowers L., LaCagnin L. B. and Colby H. D.: Metabolism of spironolactone by adrenocortical and hepatic microsomes: relationship to cytochrome *P*-450 destruction. *J. Pharmac. Exp. Ther.* **236** (1986) 675–680.
14. Mejer L. E. and Blanchard R. C.: Fluorometric determination of plasma 11-hydroxycorticosteroids II. Studies on the specificity of the method. *Clin. Chem.* **19** (1973) 718–724.
15. Begue R. J., Gustafsson J. Å. and Goldman Å. S.: New potent inhibitors of 3β-hydroxy-Δ^5-steroid oxidoreductase with short duration of action. *Endocrinology* **95** (1974) 238–256.
16. Eacho P. I. and Colby H. D.: Differences in microsomal steroid metabolism between the inner and outer zones of the guinea pig adrenal cortex. *Endocrinology* **116** (1985) 536–541.
17. Nebert D. W. and Gelboin H. V.: Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. *J. Biol. Chem.* **243** (1968) 6242–6249.
18. Nash T.: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* 55 (1953) 416–421.
19. Pitrolo D. A., Rumbaugh R. C. and Colby H. D.: Maturational changes in adrenal xenobiotic metabolism in male and female guinea pigs. *Drug Metab. Dispos.* 7 (1979) 52–56.
20. Omura T. and Sato R.: The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239** (1964) 2370–2388.
21. Kominani S., Shinzawa K. and Takemori S.: Immunochemical studies on cytochrome *P*-450 in adrenal microsomes. *Biochim. Biophys. Acta* 755 (1983) 163–169.
22. Laemmli V. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **22** (1970) 680–685.
23. Towbin H., Staehelin T. and Gordon J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natn. Acad. Sci. U.S.A.* **76** (1979) 4350–4357.
24. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
25. Meschter C. L., Mico B. A., Mortillo M., Feldman D., Garland W. A., Riley J. A. and Kaufman L. S.: A 13-week toxicologic and pathologic evaluation of prolonged cytochromes *P*450 inhibition by 1-aminobenzotriazole in male rats. *Fund. Appl. Toxic.* **22** (1994) 369–381.
26. Ortiz de Montellano P. R., Mico B. A., Mathews J. M., Kunze K. L., Miwa G. T. and Lu A. Y. H.: Selective inactivation of cytochrome *P*-450 isozymes by suicide substrates. *Arch. Biochem. Biophys.* **210** (1981) 717–728.
27. Mathews J. M., Dostal L. A. and Bend J. R.: Inactivation of rabbit pulmonary cytochrome *P*-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole. *J. Pharmac. Exp. Ther.* **235** (1985) 186–190.
28. Woodcroft K. J., Szczepan E. W., Knickle L. C. and Bend J. R.: Three *N*-aralkylated derivatives of 1-aminobenzotriazole as potent and isozyme-selective, mechanism-based inhibitors of guinea pig pulmonary cytochrome *P*-450 *in vitro*. *Drug Metab. Dispos.* **18** (1990) 1031–1037.
29. Mugford C. A., Mortillo M., Mico B. A. and Tarloff J. B.: 1-Aminobenzotriazole-induced destruction of hepatic and renal cytochromes *P*-450 in male Sprague–Dawley rats. *Fund. Appl. Toxic.* **19** (1992) 43–49.