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FORMATION OF LIGAND AND METABOLITE COMPLEXES AS A MEANS FOR SELECTIVE QUANTITATION OF CYTOCHROME P450 ISOZYMES

PETER H. ROOS,* BEATA GOLUB-CIOSK, PETRA KALLWEIT, DETLEF KAUCZINSKI and WALTER G. HANSTEIN

Institute for Physiological Chemistry, Division of Bioenergetics, Ruhr-University Bochum, D-4630 Bochum, Federal Republic of Germany

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Abstract—The suitability of triacetyloleandomycin (TAO) metabolite complex formation and metyrapone binding to reduced cytochrome P450 as a means for selective isozyme quantitation has been studied. Although isozymes of both subfamilies bind metyrapone in the reduced state, selective quantitation of 2B isozymes through the metyrapone complex is possible after complex formation of P450 3A with a TAO metabolite. Thus, consecutive application of both reactions allows the spectroscopic quantitation of P450 3A and 2B isozymes. Complete conversion of P450 3A into the complex, a precondition for P450 3A quantitation, requires NADH in addition to NADPH. A precise collective quantitation of 3A + 2B isozymes as metyrapone complexes alone is not possible because the corresponding complexes possess different molar extinction coefficients, i.e 71.5 and 52 mM^{-1} cm^{-1} at 446–490 nm, respectively. The formation of the TAO complex appears to be quite specific, since it correlates well with 3A-specific enzymatic activities, i.e. TAO N-demethylation and formation of 2β-hydroxy-, 15β-hydroxy- and 6-dehydrotestosterone. P450 3A levels in liver microsomes of male rats either untreated or treated with TAO, dexamethasone (DEX), phenobarbital or hexachlorobenzene amount to 13%, 78%, 66%, 24% and 11% of total P450, respectively. Good correlation between these values and P450 3A-specific enzymatic activities is obtained. By the spectroscopic method, P450 2B isozymes could not be detected in microsomes of untreated rats. With TAO, DEX and hexachlorobenzene the microsomal 2B level is elevated to about 20% of total P450, i.e. to 0.8, 0.4 and 0.4 nmol P450/mg protein, respectively. 2B levels of about 60% of total P450 (0.75 nmol P450/mg protein) are obtained by phenobarbital treatment. Immunoblotting with anti-P450 2B shows that the ratio of expressed 2B1 and 2B2 differs depending on the type of inducer. DEX predominantly leads to induction of 2B2, which may explain the low pentoxyresorufin *O*-dependentylase activity in these microsomes.

The property of reduced cytochromes P450 to form a complex with carbon monoxide is the basis for a simple, rapid and sensitive method for the collective quantitation of P450 isozymes [1]. For the analysis of microsomal P450 profiles it is desirable to find ligands other than carbon monoxide with binding specificities for defined subpopulations of isozymes. In this respect, metyrapone has been investigated since the early eighties [2–6], although general interaction of metyrapone with microsomal P450 was observed much earlier [7–9]. Initially claimed to be specific for the phenobarbital-inducible 2B isozymes [2], metyrapone was later shown to form ligand complexes with additional isozymes [3–5]. First hints for the involvement of more than one P450 species in metyrapone binding stem from early work of Jonen *et al.* [10] who found high and low affinity binding of metyrapone to cytochromes P450, a feature later confirmed and further analysed by Mitani *et al.* [4]. The finding that K_i values for metyrapone differ considerably with regard to the substrate used [7, 9] can also be interpreted on the basis of P450 multiplicity. Treatment of rats with pregnenolone 16α-carbonitrile (PCN†) results in a 2–3-fold increase in metyrapone binding of cytochromes P450 [3, 5], amounting to about 80% of total P450. This is strong evidence for the involvement of P450 3A isozymes in metyrapone complex formation. In accordance with these results, Ritter and Franklin [11] found that 95% of total cytochrome P450 can interact with metyrapone in liver microsomes of clotrimazole-treated rats. Both PCN and clotrimazole are strong inducers for P450 3A [11–17]. Differing results concerning the molar extinction coefficient of the P450 metyrapone complex have been published [2, 3, 6, 18]. Ivanetich *et al.* [3] were apparently the first to demonstrate the contribution of isozyme complexes with different extinction coefficients to the resultant absorbance changes.

Another method for spectroscopic isozyme quantitation is the formation of metabolic intermediate complexes. The ligands of these enzymatically inactive complexes are formed by conversion of substrates such as amphetamines [18] or methylene dioxyphenyl compounds [19] to reactive

* Corresponding author: Peter H. Roos, Ruhr-Universität Bochum, Institut für Physiologische Chemie I, Abteilung Bioenergetik, Geb. MA 2/136, Universitätsstr. 150, D-4630 Bochum, Germany. Tel. (49) 234 7005290; FAX (49) 234 7002001.

† Abbreviations: DMSO, dimethyl sulfoxide; DEX, dexamethasone; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(ethanesulfonic acid); $P450_{red}$, reduced cytochrome P450; PAGE, polyacrylamide gel electrophoresis; PCN, pregnenolone 16α-carbonitrile; PROD, pentoxyresorufin *O*-dependentylase; SDS, sodium dodecyl sulfate; TAO, triacetyloleandomycin.

intermediates. The ligands in these complexes cannot be displaced by carbon monoxide [20]. Unfortunately, the isozyme specificity of most metabolite complexes is insufficiently known and cannot be reliably deduced from induction experiments [20]. Treatment of rats with triacetyloleandomycin (TAO) leads to *in vivo* formation of such a metabolic intermediate complex [21–23]. The stable complex is still present in liver microsomal preparations and its dissociation by ferricyanide oxidation allows quantitation of *in vivo* complexed P450 3A [23–25]. Quantitative P450 3A determination in microsomes of untreated rats or rats treated with non-complex-forming 3A inducers would require quantitative *in vitro* conversion of P450 3A to the metabolite complex. Data from the literature, however, show that complete conversion is only rarely achieved [25–28]. Prerequisites for isozyme-specific spectroscopic P450 quantitations are: (a) knowledge of the isozyme specificity of complex formation, (b) complete conversion of the respective isozyme to the ligand complex and (c) the availability of an accurately determined extinction coefficient for the complex. Therefore, we elaborated a method for quantitative formation of the TAO metabolite complex. Furthermore, the specificity of the reactions used for isozyme quantitation were investigated and the molar extinction coefficients of the P450 3A TAO metabolite and metyrapone complexes were determined.

To overcome the problem of overlapping isozyme specificity, our approach was to combine different methods for isozyme identification and quantitation. In this paper, we show that spectroscopic quantitation of P450 3A and 2B isozymes is possible by a combination of metabolic intermediate complex formation and subsequent ligand binding.

MATERIALS AND METHODS

Animals and animal treatment. Mature male and female Sprague–Dawley rats (200–250 g, Lippische Versuchstierzucht, Extertal, F.R.G.) were treated with P450 inducers as described by Guengerich and Martin [29] for phenobarbital and β-naphthoflavone and by Arlotto *et al.* [12] for triacetyloleandomycin. Dexamethasone (DEX), PCN and hexachlorobenzene (100 mg/kg body weight, suspended in corn oil) were given intraperitoneally by a single injection once daily for 4 consecutive days. The next day liver microsomes were prepared as described [30].

Enzyme assays. Testosterone metabolizing activity of liver microsomes was assayed in 1 mL samples containing cytochrome P450 (1 nmol), testosterone (250 μM), NADPH (1 mM), potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM) and sucrose (50 mM). The reaction was carried out at 37° for 10 min. The steroids were extracted two times with 3 mL diethylether and the combined organic phases were evaporated under nitrogen. The residue was resolved in 0.1 mL of ethanol and analysed by reversed-phase HPLC (Supelcosil RP C_{18}, 5 μm, 150 × 4.6 mm i.d.) with a linear gradient from methanol–acetonitrile–water (43:1.1:55.9) to methanol–acetonitrile–water (75:1.9:23.1). Detection was at 254 nm.

The dealkylation of pentoxyresorufin was assayed according to Pohl and Fouts [31]. The reaction mixture contained 100 mM HEPES pH 7.8, 5 mM magnesium sulfate, 1.6 mg/mL bovine serum albumin, 4 μM pentoxyresorufin and 150 nM microsomal cytochrome P450. The reaction was initiated with 500 μM NADPH and stopped after 3 min incubation at 22° with 2 mL methanol. After centrifugation for 8 min at 10,000 *g*, resorufin in the supernatant was quantitated by fluorescence spectrophotometry (excitation at 550 nm; emission at 585 nm).

TAO *N*-demethylase activity was determined in 1 mL samples containing 0.2–1.0 nmol microsomal cytochrome P450, TAO (100 μM), NADPH (350 μM), Tris–HCl (50 mM, pH 7.5), KCl (50 mM), $MgCl_2$ (10 mM), isocitrate (5 mM), isocitrate dehydrogenase (0.5 U/mL) at 37° for 4 min. The reaction was stopped with 1.5 mL 12.5% trichloroacetic acid. Formaldehyde formation was determined spectrophotometrically by the method of Werringloer [32].

Spectrophotometric quantitation of total cytochrome P450, P450 3A and P450 2B. Total cytochrome P450 was determined by the method of Omura and Sato [1] with carbon monoxide. Consecutive quantitation of P450 3A and P450 2B isozymes was done as follows: first, P450 3A was quantitatively converted into the TAO metabolite complex. For this, 3 mL samples containing 1–3 nmol cytochrome P450 (microsomes), NADPH (350 μM), NADH (300 μM), Tris–HCl (50 mM, pH 7.5), KCl (150 mM), $MgCl_2$ (10 mM), isocitrate (5 mM), isocitrate dehydrogenase (0.5 U/mL) and catalase (230 U/mL) were preincubated for 1 min at 37°. A baseline (500–400 nm) was recorded and stored (spectrum 1). The reaction was started by addition of TAO (10 μM final concentration) in dimethyl sulfoxide (DMSO) and the same volume of DMSO was added to the reference cuvette. Complex formation was either monitored by repetitive scanning in the wavelength range of 500–400 nm with cycles of 3 min, or determined by a single spectrophotometric measurement in the same wavelength range after 30 min incubation. The last spectrum was stored (spectrum 2) and spectrum 1 was subtracted from spectrum 2. For calculation an extinction coefficient of $\varepsilon = 69\ mM^{-1}\ cm^{-1}$ (456–500 nm) was used (see below). The subsequent quantitation of P450 2B isozymes as metyrapone complex is based on the methods of Jonen *et al.* [10] and Mitani *et al.* [4]. For this, the sample was reduced with sodium dithionite and supplemented with 100 μM metyrapone dissolved in 100 mM Tris–HCl, pH 7.8, 20% glycerol, 1 mM EDTA. After incubation for 4 min at 37° a spectrum was recorded (500–400 nm) and stored (spectrum 3). The 2B–metyrapone spectrum is obtained by subtraction of spectrum 2 from spectrum 3. An extinction coefficient of $52\ mM^{-1}\ cm^{-1}$ was used for quantitation of the 2B–metyrapone complex [2].

Determination of the general metyrapone binding capacity of microsomal P450 without prior formation of the TAO metabolite complex was done as follows. Microsomes were diluted with 100 mM Tris–HCl, pH 7.8, 20% glycerol, 1 mM EDTA to a P450

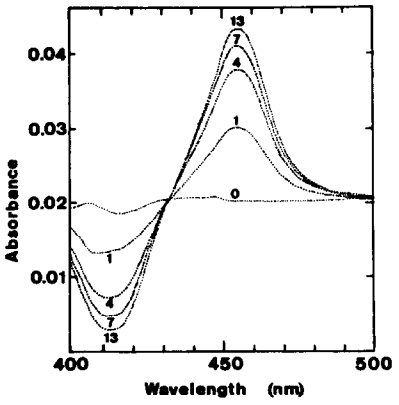

Fig. 1. Dissociation of the P450 3A–TAO metabolite complex by ferricyanide in liver microsomes of TAO-treated male rats. Microsomes were diluted with 100 mM Tris–HCl, pH 7.8, 20% glycerol, 1 mM EDTA to 0.17 mg protein/mL corresponding to 0.14 μM P450 before and 0.76 μM after treatment with ferricyanide. A baseline was recorded between 500 and 400 nm at 37° with the sample and reference cuvette containing 2 mL each of diluted microsomes. Potassium ferricyanide (1 μM) was added to the reference cuvette and the spectral changes were recorded after 1, 4, 7 and 13 min as indicated.

concentration of about 0.5 μM, reduced with sodium dithionite and supplemented with 100 μM metyrapone. A difference spectrum was recorded under the experimental conditions described above.

For complete dissociation of the P450 3A–TAO metabolite complex formed either *in vivo* or *in vitro*, the microsomes were incubated with 30 μM potassium ferricyanide for 10 min at 37°. Subsequent determinations of CO or metyrapone complexes were done as described above. Quantitation of the dissociated complex is performed by tracing a difference spectrum of an untreated against a ferricyanide-treated sample. An extinction coefficient for the P450 3A–TAO metabolite complex of $\varepsilon = 69\ mM^{-1}\ cm^{-1}$ was used (see below) for the wavelength pair 456 and 500 nm.

Determination of molar extinction coefficients. The molar extinction coefficient of the P450 3A–TAO metabolite complex was determined by partial dissociation of the complex with ferricyanide in the concentration range of 0.1–2 μM, and subsequent quantitation of liberated complex and free P450 by CO binding in the same sample. The resulting extinction data were plotted and analysed by linear regression. The extinction coefficient of the TAO metabolite complex was calculated from the slope and is based on the coefficient for the reduced cytochrome ($P450_{red}$)–CO complex (91 $mM^{-1}\ cm^{-1}$). Dissociation by ferricyanide treatment (30°) was monitored by repetitive scanning in the wavelength range of 510–400 nm. After nearly complete oxidation, taking about 10 min, the sample was reduced by sodium dithionite and the $P450_{red}$–CO complex was quantified. In the repetitive scans, a clear isobestic point at 433 nm was observed (Fig. 1).

The extinction coefficient of the P450 3A–metyrapone complex was determined as described above for the TAO metabolite complex except that metyrapone was used as ligand instead of carbon monoxide. In this case the basis for calculation is the extinction coefficient of the P450 3A–TAO metabolite complex determined as described above ($69\ mM^{-1}\ cm^{-1}$). Experimental conditions for optimal formation of the metyrapone complex are as described above.

SDS–PAGE and immunoblotting. Microsomal protein patterns were analysed by SDS–PAGE with an acrylamide concentration of 8% according to Laemmli [33] with subsequent silver staining of the protein bands [34] or transfer of the peptides to nitrocellulose by transblotting for immunostaining [35].

Purification of P450 2B and antibody production. P450 2B1 was purified according to Ryan *et al.* [36]. Prior to immunization the detergent was removed from the purified P450 preparation by chromatography on hydroxyapatite [37]. Rabbits were immunized by subcutaneous injections of purified enzymes emulsified in Freund's complete adjuvans for the first and incomplete adjuvans for the following injections. Blood was drawn from the ear vein and complete serum was used for immunoblotting.

Protein determination. The protein concentration was quantitated by the method of Lowry *et al.* [38] using bovine serum albumin for calibration.

Chemicals. Chemicals were of reagent grade and purchased from Merck (Darmstadt, F.R.G.), except for Lubrol PX, DEX, PCN, β-naphthoflavone (Sigma, Deisenhofen, F.R.G.), goat anti-rabbit

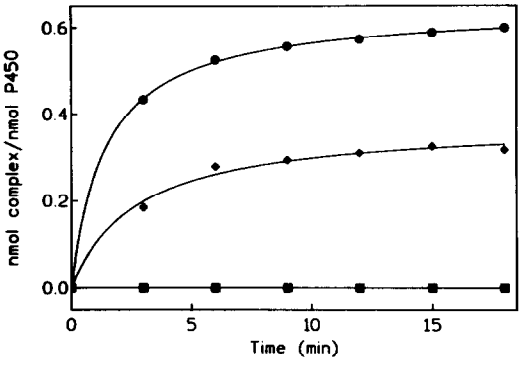

Fig. 2. Formation of the TAO metabolite complex in microsomes of DEX-treated male rats. Sample and reference cuvettes contained 2 mL each of 0.1 M Tris–HCl, pH 7.4, 20% glycerol, 1 mM EDTA, 6.25 mM glucose 6-phosphate, 7 μM $MgCl_2$, 7 μM $MnCl_2$, 1 U glucose 6-phosphate dehydrogenase, 0.31 mg microsomal protein/mL (0.58 μM P450) and either 0.8 mM NADPH (diamonds), 0.3 mM NADH (squares) or both pyridine nucleotides (circles) as indicated. The reaction was started by addition of 30 μM TAO in DMSO to the sample cuvette and addition of a respective volume of DMSO to the reference cuvette. The reaction was monitored by repetitive scanning in the wavelength range between 500 and 400 nm. The amount of complex formed is calculated by the absorbance difference 456–500 nm.

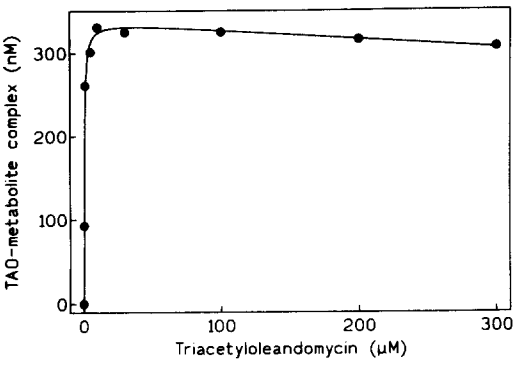

Fig. 3. Formation of the TAO metabolite complex in the concentration range of 0.2–300 μM TAO. The TAO metabolite complex was formed in liver microsomes of DEX-treated rats under the optimal conditions described in Materials and Methods. TAO concentrations as indicated. Sample: 0.5 μM P450, 0.26 mg protein/mL.

Table 1. Apparent K_M values for TAO in P450 3A complex formation obtained with microsomes of phenobarbital-, DEX- and TAO-treated male rats

Inducer	Apparent K_M (μM)	Method
PB	0.22 ± 0.055	A
PB	0.13 ± 0.024	B
DEX	0.26 ± 0.035	B
DEX	0.33 ± 0.050	C
TAO	0.69 ± 0.086	A

Values were obtained by plotting the TAO concentration against complexed P450 3A either determined directly as TAO metabolite complex (A) or indirectly as decrease in carbon monoxide (B) or metyrapone binding capacity (C).
Original data were analysed by non-linear regression to obtain the apparent K_M values. PB, phenobarbital.
Values are means ±SD.

immunoglobulin G–peroxidase conjugate (Nordic, Bochum, F.R.G.), acrylamide, *N,N'*-methylene-bis-acrylamide, *N,N,N',N'*-tetramethylethylene-diamine, phenobarbital (Serva, Heidelberg, F.R.G.), hydroxyapatite (Bio-Rad Labs, Munich, F.R.G.), 2β-hydroxytestosterone, 6β-hydroxy-testosterone and 6-dehydrotestosterone (Steraloids, Wilton, NH, U.S.A.). 15β-Hydroxytestosterone and TAO were kind gifts of Searle (Stokie, IL, U.S.A.) and Pfizer GmbH (Karlsruhe, F.R.G.), respectively.

RESULTS

Quantitative formation of the P450 3A–metabolite complex with TAO in vitro

Liver microsomes of untreated and inducer-treated rats were used to study and optimize the *in vitro* formation of the P450–TAO metabolite complex. Figure 2 shows complex formation in microsomes of DEX-treated male rats. Under the conditions described by others [26] only incomplete formation is observed. Usually about 40–50% complex is formed *in vitro* reaching the maximum after about 20 min incubation time at 37° in the presence of a NADPH-regenerating system. An incubation temperature of 37° is chosen to accelerate the reaction. This increases the initial rate of complex formation by a factor of 5.5 without altering its extent (data not shown). Complete conversion of P450 3A to the TAO metabolite complex, however, is obtained only in the presence of additional NADH, whereas NADH alone does not support complex formation (Fig. 2).

The concentrations of TAO used for *in vitro* complex formation vary between 10 and 300 μM [23–26, 39]. Titrations with TAO (Fig. 3) show that a concentration of 10 μM TAO is optimal. Apparent K_M values for TAO obtained with microsomes of rats treated with various inducers are calculated by

Table 2. Total P450, P450 3A and P450 2B content of liver microsomes of untreated and inducer-treated rats

Inducer (sex of rat)	Total P450	P450 3A (nmol/mg protein)	P450 2B
TAO (m)	4.20	3.31 (78.8)	0.83 (19.7)
TAO (m)	4.58	3.55 (77.5)	0.79 (17.0)
DEX (m)	1.90	1.25 (65.7)	0.38 (20.0)
PB (m)	1.25	0.26 (20.5)	0.79 (63.1)
PB (m)	1.34	0.37 (27.6)	0.71 (53.0)
HCB (m)	1.80	0.20 (10.9)	0.40 (22.0)
UT (m)	0.90	0.11 (12.7)	≤0.01
TAO (f)	1.75	0.93 (53.1)	0.78 (44.6)
PB (f)	1.48	0.16 (10.7)	0.65 (43.7)

Total P450 was determined as carbon monoxide complex [1], P450 3A as TAO metabolite complex and P450 2B as metyrapone complex after formation of the P450 3A–TAO metabolite complex (see Materials and Methods). PB, phenobarbital; HCB, hexachlorobenzene; UT, untreated.
Values in parentheses are % of total P450.

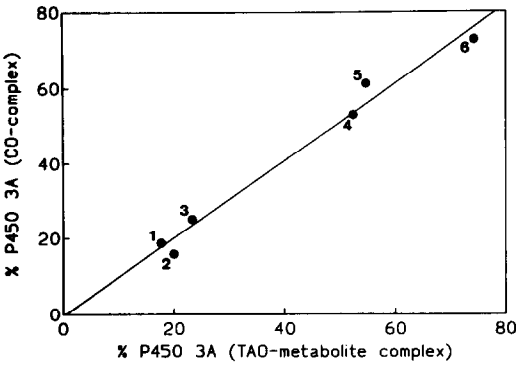

Fig. 4. Correlation of P450 3A determinations by (a) quantitation of the TAO metabolite complex and (b) carbon monoxide binding capacity before and after complex formation. The quantitations were performed as described in Materials and Methods. The data points were obtained with microsomes of (1) untreated male, (2) phenobarbital-treated male, (3) PCN-treated male, (4) TAO-treated female, (5) DEX-treated male and (6) TAO-treated male rats. $r^2 = 0.979$, slope: 1.03 ± 0.07.

non-linear regression analysis and are in the range of 0.22–0.69 μM (Table 1).

Spectroscopic quantitation of P450 3A through TAO metabolite complex

By the optimized method for complex formation used in Fig. 2, we quantified P450 3A in microsomes of adult male and female rats that were either untreated or treated with various inducers (Table 2). In microsomes of untreated male rats P450 3A constitutes about 12% of total P450, i.e. 0.1 nmol P450/mg protein. 3A levels are elevated by treatment of the rats with hexachlorobenzene, phenobarbital, DEX and TAO, the latter being the most potent inducer examined here. Compared to untreated rats, TAO leads to a 30-fold increase in the P450 3A level, i.e. 3.4 nmol P450 3A/mg protein. The 3A inducing potency follows the order TAO > DEX > phenobarbital > hexachlorobenzene.

Complexed P450 can be quantified either directly by spectroscopic measurement of complex formation at 456 nm, or indirectly by determination of CO complexation before and after TAO metabolite complex formation. Good agreement of the values obtained by the two different methods is obtained (Fig. 4). *In vivo* formation of the P450 3A–TAO metabolite complex often leads to complete conversion of the P450 3A present, but sometimes considerable amounts of uncomplexed P450 3A can be detected in the microsomal preparation so that the amount of complex formed *in vitro* exceeds that formed *in vivo* (data not shown). This fact should be considered when the P450 3A content is determined by this method. Exact and reproducible results for quantitation require the presence of catalase in the reaction mixture. Peroxide-dependent P450 destruction as determined by decreased ability to form a $P450_{red}$–CO complex is observed by incubation of microsomes in the presence of NADPH and absence of substrate (Table 3). This process is completely prevented by catalase. Azide inhibition of the catalase activity abolishes its protective effect indicating that peroxide is responsible for P450 destruction. Furthermore, superoxide dismutase does not exhibit any preventive effect on P450 destruction (Table 3). The extent of peroxide-caused destruction is different in microsomes of rats treated with various inducers, i.e. it is dependent on the microsomal P450 composition (Table 4). The high level of destruction in microsomes of dexamethasone- and TAO-treated rats implies that P450 3A may be directly involved in this process. This is supported by a multiple linear regression analysis comparing P450 3A and 2B content with P450 destruction. The results show good correlation ($r^2 = 0.96$) mainly with the P450 3A content and with a minimal contribution of P450 2B.

Table 3. Peroxide-dependent P450 destruction caused by incubation of microsomes with NADPH in the absence of substrate

Microsomal sample +	% of destroyed cytochrome P450	N
NADPH	30.5 ± 3.4	6
NADPH + catalase	4.0 ± 3.0	4
NADPH + catalase + azide	41.4 ± 11.9	3
NADPH + superoxide dismutase	31.0 ± 11.2	2

Liver microsomes of phenobarbital-treated rats were incubated for 30 min at 37° in the presence of the compounds indicated. NADPH, 800 μM; catalase, 480 U/mL; superoxide dismutase, 60 U/mL; sodium azide, 1 mM. Buffer: 100 mM Tris–HCl, pH 7.4, 20% glycerol, 1 mM EDTA. P450 concentration, 0.85 μM. Specific P450 content, 1.5 nmol P450/mg microsomal protein. Intact cytochrome P450 was quantitated as carbon monoxide complex [1].

Values are means ± SD. N, number of experiments.

Table 4. NADPH-dependent P450 destruction in microsomes: the extent of destruction is dependent on the microsomal cytochrome P450 pattern

Inducer	% of destroyed cytochrome P450	N
Phenobarbital	30.5 ± 3.4	6
DEX	57.7 ± 3.0	4
TAO	59.6 ± 0.1	3
Hexachlorobenzene	43.9 ± 8.8	4
Untreated	35.7 ± 3.4	3

Liver microsomes of male rats either untreated or treated with the inducers indicated were incubated ±800 μM NADPH for 30 min at 37°. Sample volume, 3 mL. P450 concentration, 0.8–1.0 μM. Cytochrome P450 was determined in the sample (+NADPH) and the reference (−NADPH) by the method of Omura and Sato [1]. The amount of peroxidatively destroyed P450 in the samples is expressed as per cent of the respective references.

Values are means ± SD. N, number of determinations.

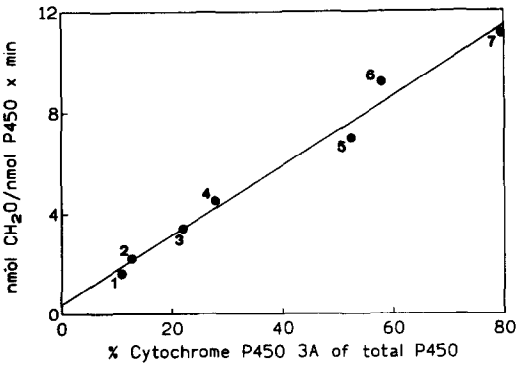

Fig. 5. TAO N-demethylation as a function of the P450 3A content in rat liver microsomes. P450 3A quantitation through formation of the TAO metabolite complex and TAO N-demethylation were performed as described in Materials and Methods. The data points were obtained with microsomes of (1) hexachlorobenzene-treated male, (2) untreated male, (3) phenobarbital-treated male, (4) PCN-treated male, (5) TAO-treated female, (6) DEX-treated male and (7) TAO-treated male rats. $r^2 = 0.983$.

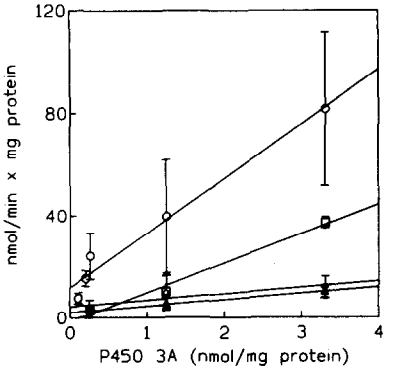

Fig. 6. Microsomal testosterone hydroxylation as a function of the P450 3A content. Quantitation of P450 3A through formation of the TAO metabolite complex and testosterone hydroxylation was performed as described in Materials and Methods. Samples: liver microsomes of untreated rats or rats treated with phenobarbital, DEX, TAO or hexachlorobenzene. 6β-Hydroxytestosterone (circles); 2β-hydroxytestosterone (squares); 15β-hydroxytestosterone (diamonds); 6-dehydrotestosterone (triangles).

Isozyme specificity of complex formation

In order to ascertain the isozyme specificity of the TAO metabolite complex, the extent of complex formation was compared with enzymatic activities specific for the P450 3A family. In the multi-step process of TAO metabolite complex formation, P450 3A is involved in two steps of the reaction sequence, i.e. the initial N-demethylations and the ultimate formation of the complex proper [21]. The enzyme components responsible for the intermediate step(s) are not clearly defined up to now. As the rate of TAO demethylation is initially independent of the metabolite complex formation, the former reaction can be used as marker activity for P450 3A. Figure 5 shows that there is a strong correlation between liver microsomal TAO N-demethylation activity and P450 3A content of rats treated with various inducers. Furthermore, good correlations are obtained between the amount of TAO metabolite complex and the rate of formation of 6β-hydroxytestosterone, 2β-hydroxytestosterone, 15β-hydroxytestosterone and 6-dehydrotestosterone (Fig. 6), four predominantly P450 3A-dependent activities [40–42].

Isozyme specificity of cytochrome P450 quantitation by metyrapone binding

The initially assumed absolute specificity of metyrapone for P450 2B isozymes [2] is not supported by results obtained by others [3, 5, 11] and by ourselves. If the method of Luu-The *et al.* [2] is used for 2B quantitation, microsomal preparations obtained from rats treated with the P450 3A inducers DEX or TAO appear to contain P450 2B levels exceeding the total P450 content (Table 5). These

Table 5. Spectroscopic quantitation of P450 2B isoenzymes in liver microsomes of rats by the method of Luu-The *et al.* [2]: complex formation with metyrapone

Inducer (sex of rat)	Total P450 (nmol P450/mg protein)	P450 2B*	% P450 2B of total P450*	N
UT (m)	0.86 ± 0.16	0.13 ± 0.03	14.7 ± 1.0	4
UT (f)	1.19 ± 0.10	0.31 ± 0.18	29.9 ± 16.8	5
PB (m)	1.56 ± 0.17	1.21 ± 0.21	77.5 ± 8.5	4
βNF (m)	0.93 ± 0.13	0.22 ± 0.09	23.9 ± 10.1	3
HCB (m)	1.32 ± 0.69	0.43 ± 0.18	33.6 ± 4.0	2
DEX (m)	2.06 ± 0.25	2.51 ± 0.48	122 ± 12.7!	6
TAO (m)	3.56 ± 0.48	4.23 ± 0.36	121 ± 9.9!	6

P450 2B was quantitated by metyrapone binding as described by Luu-The *et al.* [2] in liver microsomes of rats treated with various inducers. UT, untreated; PB, phenobarbital, βNF, β-naphthoflavone; HCB, hexachlorobenzene.

Values are means ± SD. N, number of determinations.

* These data are overestimates due to the presence of P450 3A (see text).

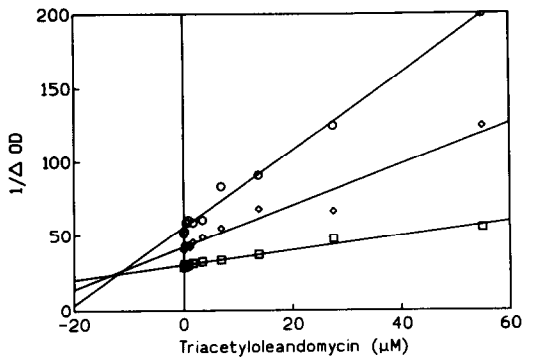

Fig. 7. Competitive inhibition of P450 metyrapone binding by TAO. Microsomes of DEX-treated (2.5 μM P450) male rats were preincubated with TAO (dissolved in DMSO) for 5 min at 20° and reduced with sodium dithionite. Difference spectra were recorded after addition of 5 (circles), 10 (diamonds) or 30 (squares) μM metyrapone. The resulting metyrapone complex was quantitated by the absorption difference (446–470 nm).

results indicate not only that P450 3A is able to form a ligand complex with metyrapone but also that the molar extinction coefficient of this complex is different from that of the 2B–metyrapone complex.

Evidence for the interaction of P450 3A with metyrapone comes from two different sets of experiments. (1) Binding of metyrapone to cytochrome P450 is competitively inhibited by TAO, a P450 3A-specific ligand (Fig. 7). (2) After P450 3A-specific formation of a TAO metabolite complex, the binding capacity of microsomal P450 for metyrapone is reduced. For example, metyrapone binding to P450 is completely abolished in microsomes of untreated male rats after formation of the TAO complex. A decrease in metyrapone binding of about 85% is found in microsomes of TAO- and DEX-treated rats (compare columns 4 in Tables 2 and 5). Residual binding sites correspond to non-3A isozymes as outlined below.

Spectroscopic quantitation of cytochrome P450 2B isozymes

Data from the literature and our own results show that members of at least two P450 isozyme subfamilies present in liver microsomes of male rats, P450 2B and P450 3A, can form metyrapone complexes in the reduced state. Nevertheless, metyrapone can be used for P450 2B quantitation after blocking the 3A isozymes by formation of a TAO metabolite complex. Consecutive application of the two methods thus allows quantitation of both groups of isozymes (Table 2). It is an open question, however, as to whether metyrapone binding is restricted to 2B and 3A isozymes. Determination of apparently P450 2B-specific enzymatic activities, i.e. pentoxyresorufin *O*-dependentylase (PROD) and testosterone 16β-hydroxylase, in microsomes of untreated and inducer-treated rats, reveals no strong correlation with the spectroscopically determined P450 2B content (Table 6).

SDS–PAGE of detergent-solubilized microsomes and subsequent immunoblotting with antibodies against P450 2B shows that the relative amounts of 2B1 and 2B2 differ with regard to the inducer used (Fig. 8). Judged from these analyses, DEX predominantly induces the 2B2 isozyme, whereas phenobarbital is a strong inducer of both isozyme forms but with a more pronounced effect on 2B1 expression. This differential inductive effect may be responsible for the observed discrepancies between P450 2B content and the corresponding enzymatic activities (see Discussion).

Determination of the molar extinction coefficients for the P450 3A–metyrapone and TAO metabolite complex

The molar extinction coefficient for the P450 3A–TAO metabolite complex formed *in vivo* by treatment of rats with TAO was determined in the following way. Microsomes containing the metabolite complex were treated with various concentrations of potassium ferricyanide in the range of 0.1–2 μM for partial dissociation of the complex. The absorbance difference due to dissociated complex was determined, the sample reduced with sodium dithionite and exposed to carbon monoxide. Then, the absorption difference of the resulting $P450_{red}$–CO complex was determined. The values were analysed by linear regression and the slope was used for calculation of the molar extinction coefficient of the 3A–TAO metabolite complex based on the value of $\varepsilon = 91\ mM^{-1}\ cm^{-1}$ for the $P450_{red}$–CO complex. By this method a value of $69.0 \pm 1.0\ mM^{-1}\ cm^{-1}$ was obtained ($r^2 = 0.9994$) (Fig. 9).

The molar extinction coefficient for the P450 3A–metyrapone complex was determined to $\varepsilon = 71.5 \pm 3.0\ mM^{-1}\ cm^{-1}$ in the same way, but using metyrapone instead of carbon monoxide. The calculation is based on the extinction coefficient of $69.0\ mM^{-1}\ cm^{-1}$ given above for the P450 3A–TAO metabolite complex.

DISCUSSION

Formation of the P450 3A–TAO metabolite complex can be used for quantitative isozyme determination, provided that complete conversion of the isozyme into the complex is achieved. Although the methods for *in vitro* TAO metabolite complex formation used by various authors are similar, published data differ with respect to the extent of the complex formed. Whereas Watkins *et al.* [24] achieve complete conversion, the data of others [25, 27, 28] indicate that only a fraction of the P450 3A present in the microsomes is complexed. Delaforge *et al.* [26] have shown that excess TAO inhibits complex formation and found the optimal concentration to be 10 μmol/L. This may explain the results of Tinel *et al.* [25] and Pershing and Franklin [23] who used TAO in concentrations of 100 and 133 μM, respectively, and achieved only partial complexation. Experiments in our laboratory have shown that complex formation is only slightly inhibited by TAO concentrations in the range of 100–300 μmol/L. However, the TAO concentration is not the only factor affecting complex formation.

Table 6. PROD and testosterone 16β-hydroxylase activity in liver microsomes of untreated and inducer-treated male rats compared to spectrophotometrically determined P450 2B content

Inducer	PROD activity (nmol resorufin/ mg protein × min)	2B content (nmol 2B/ mg protein)	PROD activity (nmol resorufin/ nmol 2B × min)	16β-Hydroxylase (nmol product/ mg protein × min)	2B content (nmol 2B/ mg protein)	16β-Hydroxylase (nmol product/ nmol 2B × min)
UT	0.04 ± 0.01	≤0.01	—	≤0.01	≤0.01	—
DEX	0.11 ± 0.01	0.38	0.28 ± 0.01	5.3 ± 0.4	0.38	14.0 ± 1.1
PB	2.18 ± 0.21	0.79	2.76 ± 0.27	15.4 ± 5.2	0.79	19.5 ± 6.5
HCB	0.34 ± 0.01	0.40	0.84 ± 0.02	1.7 —	0.40	4.4 —

Liver microsomes of male rats either untreated (UT) or treated with DEX, phenobarbital PB or hexachlorobenzene HCB were assayed for PROD and testosterone 16β-hydroxylase activity as described in Materials and Methods. The microsomal P450 2B content was determined spectrophotometrically as described in this paper.

Values of enzymatic activities are means ± SD calculated from three measurements, except for 16β-hydroxylase activity in microsomes of HCB-treated rats (N = 1).

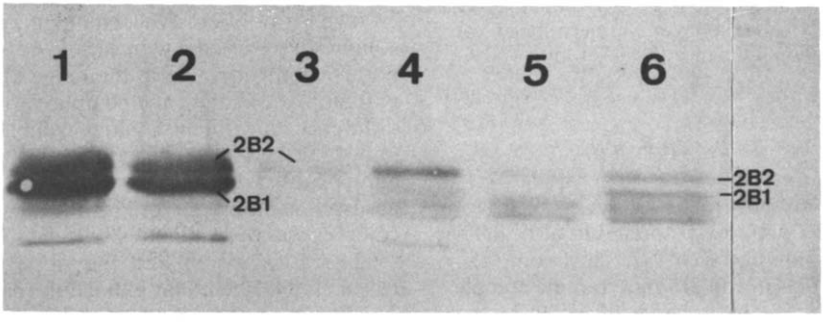

Fig. 8. Differential expression of P450 2B1 and 2B2 by treatment of rats with various inducers. Liver microsomes of either untreated or inducer-treated rats were analysed by SDS–PAGE with subsequent immunoblotting using a rabbit antiserum against P450 2B1/2B2 as described in Materials and Methods. Samples and amounts of microsomal P450 applied on SDS–PAGE (pmol): (1) phenobarbital-treated males, 4.0; (2) phenobarbital-treated females, 4.0; (3) TAO-treated males, 5.5; (4) DEX-treated males, 7.7; (5) untreated males, 4.3; (6) hexachlorobenzene-treated males, 3.9.

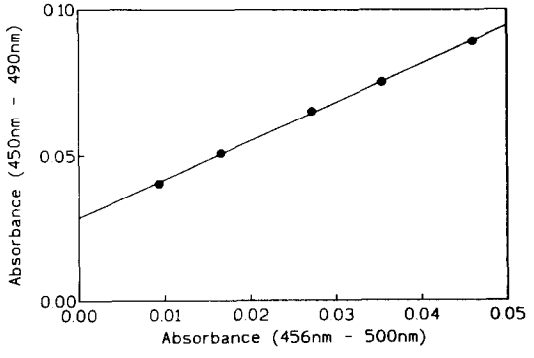

Fig. 9. Determination of the molar extinction coefficient of the P450 3A–TAO metabolite complex. Absorbance differences of the TAO metabolite complex (456–500 nm) and the P450 carbon monoxide complex (450–490 nm) were plotted. The experimental procedure is described in detail in Materials and Methods. $r^2 = 0.999$.

With the optimal TAO concentration, i.e. 10 μM [26], we achieve only about 50% complexation of P450 3A. Quantitative P450 3A–TAO metabolite complex formation, however, is obtained by addition of a further cofactor, NADH, to the reaction mixture. Using this method, the extent of complex formation amounts to about 80% of total P450 in microsomes of TAO-treated male rats, leaving only 20% for other isozymes. Further support for this quantitation method is provided by the correlation of spectroscopically determined P450 3A and TAO *N*-demethylase activity in microsomes from untreated rats and rats treated with non-complex-forming 3A inducers, like DEX and phenobarbital ($r^2 = 0.977$). The large degree of P450 3A specificity of TAO N-demethylation is evident from correlation with 3A-specific testosterone-metabolizing activities [40–42], i.e. formation of 2β-hydroxytestosterone, 15β-hydroxytestosterone and 6-dehydrotestosterone (Fig. 6). The strongest evidence for complete conversion of P450 3A comes from studies with microsomes of TAO-treated rats, when the amount of *in vitro* formed complex equals or surpasses that of *in vivo* complex.

Under our assay conditions, complex formation is

completed after about 20 min irrespective of the level of complexable P450 3A. In the literature, incubation times vary between 5 and 60 min [24, 25, 39, 43]. In a recent paper [44] maximum complex formation was obtained only after 60 min. It is likely that slow complex formation under suboptimal conditions in combination with short incubation times may be another factor leading to an underestimation of the P450 3A content determined by this technique. Errors in quantitation can apparently also result from peroxidative P450 destruction during the assay. Our observation that P450 3A is exceptionally sensitive for this process is in agreement with the results of Kitada *et al.* [45] who demonstrated preferential oxidative destruction of testosterone 6β-hydroxylase and ethylmorphine *N*-demethylase. Protection can be achieved with catalase and/or EDTA [46]. Different temperatures during complex formation, i.e. 25° or 35° [44, 47], may also contribute to the variation in the results obtained. Quantitation of P450 3A solely by determination of *in vivo*-formed complex in liver microsomes of TAO-treated rats can also result in underestimation of the isozyme level because of the presence of uncomplexed P450 3A.

The use of metyrapone for spectroscopic quantitation of cytochrome P450 isozymes has been frequently explored [2–6, 10]. The isozyme specificity of metyrapone binding, however, has not been fully clarified. As shown in this paper, the ability of P450 3A to interact with metyrapone and to form a ligand complex in the reduced state severely restricts the usefulness of the spectroscopic quantitation method for 2B isozymes as described by Luu-The *et al.* [2] for the following reasons. (a) Microsomes often contain isozymes of both subfamilies shown to bind metyrapone. For example, treatment of rats with P450 3A inducers, like DEX and clotrimazole leads also to expression of 2B isozymes [16, 48] (Table 2). The classical 2B inducer phenobarbital increases the microsomal content of P450 3A also [15, 49, 50]. Only negligible levels of 2B isozymes can be detected in liver microsomes of untreated male rats [51] amounting to about 20 pmol/mg protein in Sprague–Dawley rats [52]. Thus, in these microsomes metyrapone binding is essentially due to P450 3A. (b) Due to the very different extinction coefficients of the 2B- and 3A-metyrapone complexes (52 [2] and $71.5 \pm 3.0\ mM^{-1}\ cm^{-1}$, respectively) not even a collective quantitation of both isozyme groups is possible. (c) 2B and 3A isozymes bind metyrapone with about the same affinity [5] (unpublished data). Thus, isozyme determinations at different metyrapone concentrations [4] are not useful for these P450 species.

The quantitation of 2B isozymes using metyrapone becomes possible after conversion of the 3A isozymes to a metabolic intermediate complex with TAO and the subsequent formation of the 2B–metyrapone complex. A precondition for this method is that the TAO metabolite complex is not dissociated by metyrapone. This has been shown directly by Werringloer and Estabrook [18] and Franklin [20] and confirmed by us using metyrapone concentrations of up to 300 μM (data not shown). This is as expected from the facts that (a) carbon monoxide is a stronger ligand for reduced P450 than metyrapone [10], and (b) the P450 TAO metabolite complex is stable towards carbon monoxide.

Whether metyrapone forms complexes with P450 species other than P450 2B and P450 3A is not known for certain. The conclusion of Ivanetich *et al.* [3] based on data of Luu-The *et al.* [2] that P450c (P450 1A1) is able to form a metyrapone complex has not been confirmed by others. On the contrary, Ryan *et al.* [53] could not detect metyrapone complex formation with purified P450 1A1. The inability to form a metyrapone complex has also been shown for P450 1A2 [53], 2A1 [36], 2C6 [54], 2C7, 2C11 and 2C13 [55]. In two recently published reviews P450 2C12 has been reported as an additional metyrapone complex-forming isozyme [56, 57]. This isozyme is female specific and obviously absent in the microsomes of male rats [55, 58]. Thus, the isozyme(s) responsible for the low affinity binding in microsomes of male rats remain unknown [5, 59] (unpublished data).

Increased metyrapone binding in microsomes of untreated female rats compared to that in male rats (Table 5) may well be explained by binding to the female-specific P450 2C12. Immunochemically determined microsomal 2C12 levels of female rats are in the range of 0.19–0.50 nmol/mg protein [58, 60, 61], making up about 20–50% of total P450. Thus, in liver microsomes of female rats the presence of large amounts of metyrapone bound to P450 2C12 does not allow a simple spectroscopic quantitation for 2B isozymes. As the apparent K_D values for the binding of metyrapone to reduced 2B and 2C12 differ considerably, i.e. 1.1–1.5 and 98 μM, respectively [57], a good approximation of the isozyme levels could be achieved by using different metyrapone concentrations as proposed by Mitani *et al.* [4].

With some exceptions microsomal P450 3A and 2B levels of treated and untreated rats determined with the method described here (Table 2) are similar to published data obtained by spectrophotometric or immunological methods. High 3A levels are found in liver microsomes after DEX and TAO treatment, amounting to 65% and 78% respectively, of total cytochrome P450 which is in good agreement with the results of others [39, 43, 62, 63]. Less pronounced induction of 3A isozymes is achieved with phenobarbital. In contrast to the results of others [25, 27, 44], we obtained considerable formation of a TAO metabolite complex with microsomes of untreated male rats. With a level of about 13% (0.11 nmol P450/mg protein) our value comes close to that of Guengerich *et al.* [64] obtained by immunoquantitation (17%).

Controversial results have been published on the inducibility of P450 2B isozymes by DEX. Meehan *et al.* [65] conclude from their results that DEX fails to induce 2B isozymes. In accordance with this, Ritter and Franklin [66] could not detect any PROD activity after DEX treatment. The same authors, however, observed a slight increase in testosterone 16β-hydroxylase activity compared to untreated rats. In contrast, Namkung *et al.* [48] and Yamazoe *et al.* [67] found increased activity of PROD, which is diagnostic for 2B isozymes [68–70], coinciding with

enhanced expression of P450 2B1. Our results present a further variation on this theme. After blocking the ligand binding site of P450 3A by TAO metabolite complex formation, 20% of total cytochrome P450 is still able to form a metyrapone ligand complex in microsomes of DEX-treated rats. However, microsomal PROD activity on a protein basis is only slightly enhanced compared to that of untreated rats (Table 6). These observations can be rationalized by preferential expression of the P450 2B2 isozyme as evidenced from immunoblotting experiments. In contrast to our results, Yamazoe *et al.* [67] observed similar levels of P450 2B1 and 2B2 after DEX treatment. P450 2B2 has been shown to bind metyrapone in the reduced state [53] and is known to exhibit lower enzymatic activities as compared to P450 2B1 [53, 71]. In the case of PROD, the enzymatic activities of 2B1 and 2B2 in a reconstituted system differ by two orders of magnitude [72]. These findings may explain the observed discrepancies between metyrapone binding capacity after TAO metabolite complex formation and PROD activity in microsomes of DEX-treated rats. The molecular basis for the preferential expression of P450 2B2 by DEX compared to P450 2B1 might be the presence of a glucocorticoid responsive element in the 5′-flanking region of the 2B2 gene [73].

Due to the complexity of microsomal cytochrome P450 patterns, a comprehensive qualitative and quantitative analysis requires a set of various specific methods. For routine analyses these methods should be simple and rapid. Therefore, we developed the spectrophotometric assays described here as part of an analytical network [74]. In contrast to semi-quantitative enzymatic methods, absolute levels of 2B and 3A-isozymes can be determined by this method.

A limitation of our method is that it does not discriminate between individual isozymes, i.e. 2B1 and 2B2, or 3A1 and 3A2. Knowledge of the isozyme ratios, however, is important for evaluation of the microsomes' metabolic potential, because 2B1 and 2B2 differ markedly in reaction velocities for different substrates [72] and 3A isozymes may even differ in substrate or inhibitor specificity [75–77]. Determination of the 2B1/2B2 ratio is possible by SDS–PAGE with subsequent immunoblotting. At present 3A1 and 3A2 cannot be resolved by SDS–PAGE. However, immobilized metal affinity chromatography may be able to separate distinct P450 3A isozymes [78, 79].*

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