ELSEVIER

Journal of Chromatography B, 734 (1999) 23–29

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Novel sensitive high-performance liquid chromatographic method for assay of coumarin 7-hydroxylation

Pavel Souček*

Biotransformations Group, Center of Occupational Diseases, National Institute of Public Health, 48 Šrobárova, 10 Prague 100 42, Czech Republic

Received 26 January 1999; received in revised form 29 June 1999; accepted 7 July 1999

Abstract

In this paper, a novel HPLC-based method with fluorometric detection of coumarin 7-hydroxylase is presented. The described method provides a time-effective, more sensitive and specific alternative to the previously used spectrofluorometric assay. Using the developed method, metabolism of coumarin in 11 samples of human liver microsomes was evaluated and 1790±690 pmol/min/nmol cytochrome P450 (CYP) activity was found. Kinetic parameters and linearity of coumarin 7-hydroxylation were studied in a reconstituted system consisting of recombinant CYP2A6 expressed in *Escherichia coli*, rat NADPH-CYP reductase and usual components. It was found that a 3.5 to 30 min time of incubation is suitable for estimation of coumarin 7-hydroxylase activity. Observed K_m and V_{max} values in the CYP2A6 reconstituted system were 1.48±0.37 μ*M* and 3360±180 pmol product/min/nmol CYP, respectively.

Keywords: Enzymes; Coumarin 7-hydroxylase

1. Introduction

Coumarin is a naturally occurring constituent of many plants. Metabolism of coumarin was shown to be performed by cytochrome P450 (CYP; for reference to the nomenclature see Ref. [1]) in a variety of mammals where large interspecies differences have been observed (for review see Ref. [2]). Cytochrome P450 enzymes (CYP; EC 1.14.14.1) are the principal metabolizing enzymes involved in detoxification and/or activation of compounds of both natural and synthetic origin [3]. Some of the CYP-catalyzed reactions result in formation of cytotoxic or genotoxic intermediates (for review see Ref. [3]).

CYP2A6 is the only enzyme known to date to be responsible for coumarin 7-hydroxylase activity in human liver [4,5]. Coumarin 7-hydroxylation is therefore used as a marker for activity of CYP2A6 in biological samples and the use of coumarin as an in vivo probe of CYP2A6 in humans has also been reported [6]. It was shown that coumarin 7-hydroxylase activity among individual human livers differs up to 260-fold and relative CYP2A6 content on average accounts for about 4% of total CYP content [7,8]. Genetic polymorphism reflected in three allelic variants (newly designated 2A6*1, 2A6*2, 2A6*3) was identified [9,10].

Recently, CYP2A6 was expressed in *Escherichia coli* and antibodies have been produced against recombinant CYP2A6 [11].

In this paper a novel, sensitive and rapid method

*Tel.: +420-2-6708-2711; fax: +420-2-6731-1236.
E-mail address: pavel.soucek@ecn.cz (P. Souček)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00325-4

for assay of coumarin 7-hydroxylation is described. This method may serve as an important tool in further studies of the role of CYP2A6 in the metabolism of chemicals, pro-carcinogen activation, and in assessment of the biological relevance of CYP2A6 genetic polymorphisms.

2. Experimental

2.1. Chemicals

Coumarin, 7-hydroxycoumarin (umbelliferone), components of the reaction mixtures, and other reagents were purchased from Sigma (St. Louis, MO, USA). Acetonitrile LiChrosolv (for chromatography) was a product of Merck (Darmstadt, Germany). Recombinant CYP2A6 was expressed in *Escherichia coli* and purified as described [11]. Rat NADPH-CYP reductase was purified from liver microsomes of phenobarbital-treated rats by the method of Yasukochi and Masters [12] as modified by Ref. [13].

2.2. Apparatus

The high-performance liquid chromatography (HPLC) apparatus consisted of a pump (LCP4000, ECOM, Prague, Czech Republic) coupled with a programmable fluorescence detector HP 1046A (Hewlett-Packard, USA). 7-Hydroxycoumarin was detected at 338 nm excitation and 458 nm emission wavelength with the photomultiplier tube (PMT) gain of the fluorescence detector set to 8, response time was 1 s and flash frequency 1.25 W/55 Hz. An isocratic mobile phase consisting of 30 m*M* sodium borate (pH 8.0)–acetonitrile (80:20) was run at flow-rate of 1 ml/min on a Nucleosil 100, 5 μm, C_{18}, (250×4 mm) column (Macherey-Nagel, Dueren, Germany). Peak parameters were evaluated using a threshold limit 1 mV and range up to 1000 mV by CSW v1.7 software produced by DataApex (Prague, Czech Republic). Fluorescence at 338/458 nm excitation/emission wavelength was also measured by spectrofluorimeter from Aminco-Bowman (Silver Spring, MD, USA).

2.3. Human liver donors

Human liver samples were obtained from the Transplantation Center (IKEM, Czech Republic) from donors who died accidentally as a result of brain injury. Liver samples were obtained at most 30 min after death and were stored in liquid nitrogen until microsomes were isolated. Some of the donors were submitted to therapy including antibiotics, mannitol, or hormones for 24 to 48 h before death. Microsomes were prepared according to van der Hoeven and Coon [14] and stored in aliquots at −80°C.

2.4. Assay of coumarin 7-hydroxylase activity

The CYP2A6 reconstituted system contained 0.5 ml of the following mixture: 25 pmol of CYP2A6, 75 pmol of rat NADPH-CYP reductase, 30 μ*M* L-α-dilauroyl-*sn*-3-phosphocholine, 50 m*M* potassium phosphate buffer (pH 7.4), 20 μ*M* coumarin (0.2–50 μ*M* in kinetic study), and NADPH-generating system (final concentrations: 10 m*M* $MgCl_2$, 10 m*M* glucose-6-phosphate, 1 m*M* NADP, 0.5 U/ml glucose-6-phosphate dehydrogenase). In experiments with human liver microsomes, 100 pmol of total CYP was used in a total volume of 0.5 ml of 50 m*M* potassium phosphate buffer (pH 7.4), 20 μ*M* coumarin and NADPH-generating system described above. After a 3-min preincubation at 37°C, the reaction was started with the addition of the NADPH-generating system and proceeded for 15 min (10 min in kinetic study; 3.5–30 min in reaction linearity study) at 37°C with shaking. Blank samples contained all components except the NADPH-generating system which was added after termination of the reaction.

2.5. Standards

Stock solutions of 7-hydroxycoumarin were prepared by dissolving 16.2 mg of 7-hydroxycoumarin in 10 ml of methanol to make a 10 m*M* solution. Separate solutions for calibration curve (25, 100, 250, 500, 750 and 1000 μ*M*) and quality control samples (50, 200 and 600 μ*M*) were prepared by serial dilutions of stock solutions with distilled

water. These solutions were stored at 4°C and were stable for at least one month.

2.6. Calibration curves

The calibration curve was prepared by incubation of 250, 1000, 2500, 5000, 7500 and 10 000 pmol of 7-hydroxycoumarin with complete incubation mixture in the absence of the NADPH-generating system which was added after termination of the reaction. Quality control samples used for study of precision and accuracy contained 500, 2000 and 6000 pmol of 7-hydroxycoumarin and were prepared and processed in the same way as calibration standards.

2.7. Sample processing

The reaction was terminated by addition of 50 μl of 0.3 *M* perchloric acid. Samples were briefly vortexed and neutralized by 50 μl of 1 *M* NaOH. After vortexing, samples were centrifuged at 4000 *g* (Hermle 360K centrifuge, Germany) for 2 min. HPLC analysis was performed using a 5-μl loop. Under the described conditions, the retention time of 7-hydroxycoumarin was 2.49 min. In the experiment designed to compare the results of HPLC and spectrofluorimetric methods, a set of duplicate samples was prepared. One set was processed as described above. A 1-ml volume of dichloromethane was added to samples of the second set, samples were vortexed for 15 s, and centrifuged at 4000 *g* for 5 min. A 0.5-ml aliquot of organic phase was extracted into 3 ml of 30 m*M* sodium borate (pH 9.0), and fluorescence (excitation 338 nm/emission 458 nm) was measured in borate–aqueous phase by the spectrofluorimeter.

3. Results and discussion

3.1. Chromatography

Typical chromatograms of blank and sample of 7-hydroxycoumarin in human liver microsomes are shown in Fig. 1. No interfering peaks were detected under the described conditions.

3.2. Linearity and limit of quantitation

A good linearity ($r=0.9993\pm0.0004$) within the concentration range 250 to 10 000 pmol of 7-hydroxycoumarin was found. This linear relationship was demonstrated by the statistical analysis of linear regression model of $y=a+bx$ (where x represents pmol of 7-hydroxycoumarin per sample and y represents peak height). Mean values±SD for the slope and intercept ($n=10$) were 0.0122 ± 0.0024 and -0.5098 ± 0.9181, respectively. The mean of the difference between true and back-calculated concentration of the calibration standards was 2.9% (range 0.7 to 15.4%); this indicates the suitability of the calibration model. The criteria used to estimate the limit of quantitation (LOQ) were maximal intra- and inter-day variation in accuracy and precision of 20%. The LOQ was estimated to be 150 pmol of 7-hydroxycoumarin per 0.5 ml sample.

3.3. Precision and accuracy

Six sets of quality control samples (500, 2000 and 6000 pmol of 7-hydroxycoumarin) were analyzed with calibration standards on one day (intra-day precision and accuracy). Inter-day precision and accuracy was evaluated by analysis of a set of calibration and quality control samples on six separate days. The precision was better than 11.6% and the inaccuracy did not exceed 10% at all concentrations of quality control samples (Table 1).

3.4. Application

Metabolism of coumarin was assayed in human liver microsomes by HPLC. Results of coumarin 7-hydroxylation assay in human liver microsomes obtained using this method (Table 2; 1790±690 pmol/min/nmol CYP, $n=11$) were higher than some of the previously reported data but comparable to the turnovers seen in CYP2A6 reconstituted system (Table 2; 4530 pmol/min/nmol CYP). It was reported that coumarin 7-hydroxylase activity assayed in 30 samples of human liver microsomes of Caucasian subjects ranged from 5 to 97 pmol/min/mg of protein [7]. These data were obtained by the method described in Refs. [15,16] based on the original spectrofluorometric assay [17]. In the study

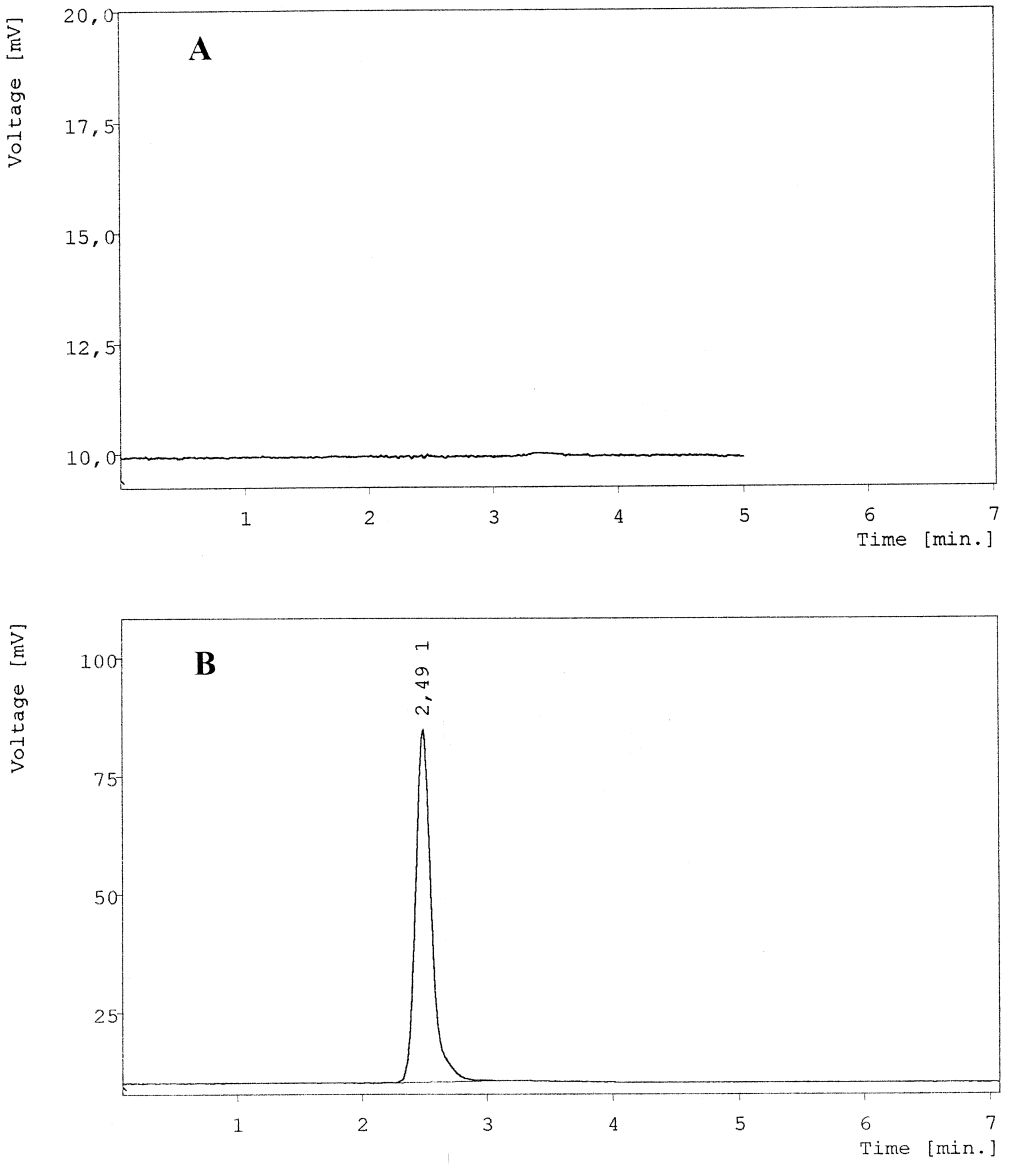

Fig. 1. Chromatograms of human liver microsomes incubated with 20 μ*M* coumarin in the absence (A) or presence (B) of NADPH-generating system. For incubation conditions see Experimental.

of Shimada et al., the incubation of 1 mg of microsomal protein with 50 μ*M* coumarin in similar buffer and NADPH-generating system as described here but with 30 min of incubation was performed [7]. The total CYP content in the mentioned samples varied from 240 to 620 pmol/mg of protein [7] and therefore the amount of microsomal CYP added per 1 ml of reaction mixture was similar to that used in the study described here. Also the data of Yamano et al. (13–1350 pmol/min/mg, $n=12$) [5], Forrester et al. (13–69 pmol/min/mg, $n=12$) [18], Nakajima et al. (0–320 pmol/min/mg, $n=16$) [19], and Nunoya et al. (0–700 pmol/min/mg, $n=20$) [20], seemed to be relatively lower that the data obtained here. On the other hand, there were also reports indicating higher activities: Yun et al. (14–2300 pmol/min/

Table 1
Accuracy and precision study of coumarin 7-hydroxylase assay

	Calculated amount[b]	Accuracy (%)[c]	Precision (%)[d]
Intra-day (n=6)			
Calibration standards[a]			
250	265.4±49.9	6.1	18.8
1000	991.9±66.3	−0.8	6.7
2500	2453.1±90.6	−1.9	3.7
5000	4918.3±200.2	−1.6	4.1
7500	7680.9±184.0	2.4	2.4
10 000	9903.8±176.5	−1.0	1.8
Quality control samples[a]			
500	519.6±60.4	3.9	11.6
2000	1965.8±72.4	−1.7	3.7
6000	5745.6±311.2	−4.2	5.4
Inter-day (n=6)			
Calibration standards[a]			
250	288.5±39.1	15.4	13.5
1000	986.8±61.2	−1.3	6.2
2500	2465.4±59.3	−1.4	2.4
5000	4925.4±135.4	−1.5	2.7
7500	7606.9±167.1	1.4	2.2
10 000	9933.0±149.2	−0.7	1.5
Quality control samples[a]			
500	549.9±57.4	10.0	10.4
2000	2000.2±45.5	0.1	2.3
6000	5861.7±248.7	−2.3	4.2

[a] Amounts of 7-hydroxycoumarin added per 0.5 ml sample in pmol.
[b] Mean±SD in pmol.
[c] Defined as: [(measured conc.−target conc.)/target conc.]×100%.
[d] Assessed by expressing the standard deviation of the measurement as a percentage of the mean value.

nmol CYP, $n=20$) [15], Koenigs et al. (640–22 580 pmol/min/nmol CYP, $n=12$) [21]; and Draper et al. (179–2470 pmol/min/mg, $n=11$) [22].

It was of interest to explore the source of the difference seen in comparison with the data reported before. It seemed that this discrepancy could be explained by longer incubation time or higher concentration of substrate used previously [7]. Enzyme kinetics of CYP2A6 where different amounts of coumarin (0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 μM) were used in 10 min of incubation showed that the activity of coumarin hydroxylase does not greatly differ in the range from 1 to 50 μM coumarin (Fig. 2). Kinetic parameters of coumarin metabolism analyzed in this study were found to be very similar to those reported for human liver [7] or CYP2A6 expressed in the baculovirus system [23]. Another experiment showed that coumarin 7-hydroxylation is independent of incubation time up to 30 min (Table 3). In this experiment, times of incubation 3, 5, 10, 15, 20 to 30 min were used. Thus, by the above mentioned experiments performed using the HPLC method the reason for discrepancy was not found.

In order to exclude the possibility that differences have arisen by different processing of the samples, results were compared to data obtained with the original spectrofluorometric method [15]. The comparison of metabolism of coumarin in human liver microsomes showed almost identical results by the use of both methods (2.36 nmol of product/min/nmol CYP by HPLC method; 2.53 nmol of product/min/nmol CYP by original method). The results

Table 2
Metabolism of coumarin in human liver microsomes and CYP2A6 reconstituted system (analysis was performed using the HPLC method). Samples were analyzed in duplicates with variations less than 5%

Human liver microsomes No.[a]	Activity (pmol of product/min/nmol CYP)
H2	2520
H4	2490
H6	2610
H7	2040
H8	1450
H9	2200
H10	1380
H11	1920
H12	1140
H13	380
H14	1530
Mean (n=11)	1790±690
CYP 2A6 reconstituted system[b]	4530

[a] Incubation mixture contained 100 pmol of total CYP in 0.5 ml of 50 m*M* potassium phosphate buffer (pH 7.4), 20 μ*M* coumarin and NADPH-generating system. Time of incubation was 15 min.

[b] Reconstituted system contained: 25 pmol CYP2A6, 75 pmol CYP reductase, 30 μ*M* L-α-dilauroyl-*sn*-3-phosphocholine, 50 m*M* potassium phosphate, 20 μ*M* coumarin and NADPH-generating system in a total volume of 0.5 ml. Incubation proceeded for 15 min.

suggest that the difference in turnovers found in the literature is not linked to time of incubation, substrate concentration and sample processing after incubation. The data presented here also show that time-effective and highly specific HPLC-based method may be used where the spectrofluorometer is not available or where rapid and specific assay is required.

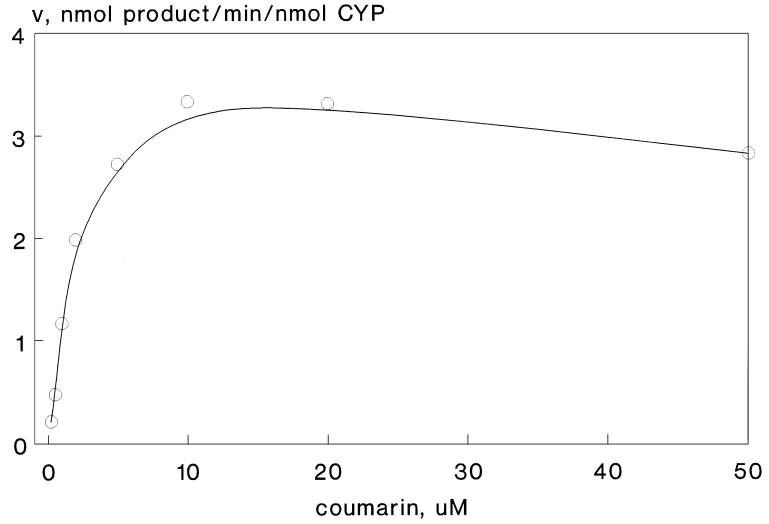

Fig. 2. Enzyme kinetics of coumarin 7-hydroxylation in CYP2A6 reconstituted system. CYP2A6 reconstituted system contained 0.5 ml of the following mixture: 25 pmol of CYP2A6, 75 pmol of rat CYP reductase, 30 μ*M* L-α-dilauroyl-*sn*-3-phosphocholine, 50 m*M* potassium phosphate buffer (pH 7.4), 0.2, 0.5, 1, 2, 5, 10, 20 or 50 μ*M* coumarin and NADPH-generating system (10 m*M* $MgCl_2$, 10 m*M* glucose-6-phosphate, 1 m*M* NADP, 0.5 U/ml glucose-6-phosphate dehydrogenase). Incubation proceeded for 10 min and 7-hydroxycoumarin was determined using HPLC method as described in Experimental. All assays were performed in duplicates with variations less than 5%. K_m=1.48±0.37 μ*M*, V_{max}=3360±180 pmol product/min/nmol CYP.

Table 3
Linearity of coumarin 7-hydroxylation in CYP 2A6 reconstituted system (analysis was performed using HPLC method). Samples were analyzed in duplicates with variations less than 5%

Incubation time (min)	Activity[a] (pmol product/min/nmol CYP)
3.5	4620
5	4450
10	4610
15	4590
20	4620
30	4430

[a] Reconstituted system contained: 25 pmol CYP 2A6, 75 pmol CYP reductase, 30 μ*M* L-α-dilauroyl-*sn*-3-phosphocholine, 50 m*M* potassium phosphate, 20 μ*M* coumarin and NADPH-generating system in a total volume of 0.5 ml. Incubation proceeded for 3.5 to 30 min.

4. Conclusions

The novel HPLC-based method presented here is valid for estimation of coumarin 7-hydroxylation in both biological samples (e.g., liver microsomes) and CYP reconstituted systems. A 10-min incubation time for the assay of coumarin 7-hydroxylation is recommended. Shorter incubation times would provide comparable results but might cause higher variation when large series of samples should be analyzed. The described HPLC method may prove to be useful for further analysis of coumarin metabolism by biotransformation enzymes as 3-hydroxy metabolite of coumarin formation by allellic variant of CYP2A6 (Leu160His) was recently reported [24].

Acknowledgements

This work was supported by a grant from the Internal Grant Agency of Czech Ministry of Health, grant IGA 3505-3.

References

[1] D.R. Nelson, L. Koymans, T. Kamataki, J.J. Stegeman, R. Feyereisen, D.J. Waxman, M.R. Waterman, O. Gotoh, M.J. Coon, R.W. Estabrook, I.C. Gunsalus, D.W. Nebert, Pharmacogenetics 6 (1996) 1–42.
[2] P. Fernandez-Salguero, F.J. Gonzalez, Pharmacogenetics 5 (1995) S123–128.
[3] F.P. Guengerich, in: P.R. Ortiz de Montellano (Ed.), Cytochrome P450 – Structure, Mechanism and Biochemistry, Plenum Press, New York, 1995, pp. 473–535.
[4] J.S. Miles, A.W. McLaren, L.M. Forrester, M.J. Glancey, M.A. Lang, C.R. Wolf, Biochem. J. 267 (1990) 365–371.
[5] S. Yamano, J. Tatsuno, F.J. Gonzalez, Biochemistry 29 (1990) 1322–1329.
[6] S. Cholerton, M.E. Idle, A. Vas, F.J. Gonzalez, J.R. Idle, J. Chromatogr. 575 (1992) 325–330.
[7] T. Shimada, H. Yamazaki, M. Mimura, Y. Inui, F.P. Guengerich, J. Pharm. Exp. Ther. 270 (1994) 414–423.
[8] T. Shimada, H. Yamazaki, F.P. Guengerich, Xenobiotica 26 (1996) 395–403.
[9] P. Fernandez-Salguero, S.M.G. Hoffman, S. Cholerton, H. Mohrenweiser, H. Raunio, A. Rautio, O. Pelkonen, J. Huang, W.E. Evans, J.R. Idle, F.J. Gonzalez, Am. J. Hum. Genet. 57 (1995) 651–660.
[10] M. Oscarson, H. Gullsten, A. Rautio, M.L. Bernal, B. Sinues, J.H. Dahl, J.H. Stengard, O. Pelkonen, H. Raunio, M. Ingelman-Sundberg, FEBS Lett. 438 (1998) 201–205.
[11] P. Souček, Arch. Biochem. Biophys., in press.
[12] Y. Yasukochi, B.S.S. Masters, J. Biol. Chem. 251 (1976) 5337–5344.
[13] F.P. Guengerich, in: A.W. Hayes (Ed.), Principles and Methods of Toxicology, Raven, New York, 1994, pp. 1259–1313.
[14] T.A. van der Hoeven, M.J. Coon, J. Biol. Chem. 249 (1974) 6302–6310.
[15] C.H. Yun, T. Shimada, F.P. Guengerich, Mol. Pharmacol. 40 (1991) 679–685.
[16] H. Yamazaki, M. Mimura, C. Sugahara, T. Shimada, Biochem. Pharmacol. 48 (1994) 1524–1527.
[17] W.F. Greenlee, A. Poland, J. Pharm. Exp. Ther. 205 (1978) 596–605.
[18] L.M. Forrester, C.J. Henderson, M.J. Glancey, D.J. Back, K.B. Park, S.E. Ball, N.R. Kitteringham, A.W. McLaren, J.S. Miles, P. Skett, C.R. Wolf, Biochem. J. 281 (1992) 359–368.
[19] M. Nakajima, T. Yamamoto, K.-I. Nunoya, T. Yokoi, K. Nagashima, K. Inoue, Y. Funae, N. Shimada, T. Kamataki, Y. Kuroiwa, Drug Metab. Dispos. 24 (1996) 1212–1217.
[20] K.-I. Nunoya, T. Yokoi, K. Kimura, T. Kodama, M. Funayama, K. Inoue, K. Nagashima, Y. Funae, N. Shimada, C. Green, T. Kamataki, J. Pharm. Exp. Ther. 277 (1996) 768–774.
[21] L.L. Koenigs, R.M. Peter, J.T. Stella, A.E. Rettie, W.F. Trager, Drug Metab. Dispos. 25 (1997) 1407–1415.
[22] A.J. Draper, A. Madan, A. Parkinson, Arch. Biochem. Biophys. 341 (1997) 47–61.
[23] C.J. Patten, T.J. Smith, S.E. Murphy, M.-H. Wang, J. Lee, R.E. Tynes, P. Koch, C.S. Yang, Arch. Biochem. Biophys. 333 (1996) 127–138.
[24] H. Hadidi, K. Zahlsen, J.R. Idle, S. Cholerton, Food Chem. Toxicol. 35 (1997) 903–907.