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Determination of tetrahydrothiophene formation as a probe of in vitro busulfan metabolism by human glutathione *S*-transferase A1-1: use of a highly sensitive gas chromatographic–mass spectrometric method

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Abstract

A method for the sensitive determination of tetrahydrothiophene (THT) in cytosolic incubation mixtures was developed. Busulfan conjugation with glutathione was predominantly catalysed by glutathione *S*-transferase A1-1 (GST A1-1) and THT was released from the primary metabolite by alkalization. After liquid–liquid extraction using *n*-pentane separation and quantification of the product was performed by gas chromatography with a mass-selective detector. The method showed good sensitivity, accuracy and reproducibility with a detection limit of 2 ng ml^{-1} and a limit of quantification of 5 ng ml^{-1}. The suitability of the method is shown for enzyme kinetic studies in human liver cytosol as well as for determination of GST A1-1 activity.

Keywords: Tetrahydrothiophene; Busulfan; Glutathione *S*-transferase A1-1

1. Introduction

Busulfan is an antineoplastic agent, which is used in the setting of bone marrow transplantation. High interindividual variability in pharmacokinetics has been observed in paediatric and adult patients [1], in particular with high-dose busulfan [2–4]. Thus, variability of plasma concentration following high dose busulfan may result in variable outcome. One particular problem is development of veno-occlusive disease (VOD), which is a serious complication in preconditioning treatment with busulfan-containing regimens. VOD is associated with high mortality [5–7] and several recent publications point to the possibility that busulfan induced VOD is concentration-dependent [8,9]. A wide variety of causal relations for concentration-related VOD is given in the literature ranging from the absorption of the drug [10], drug interactions [11], or alterations in first-pass clearance based on the biotransformation of the drug as busulfan is extensively metabolized in the

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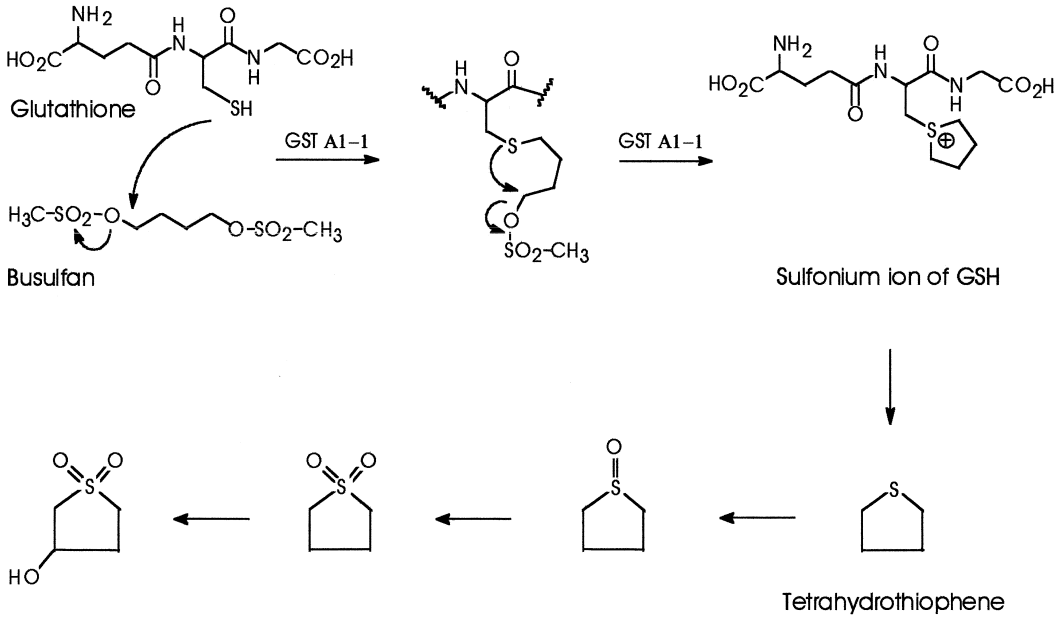

Fig. 1. Metabolic pathway of busulfan via conjugation with glutathione to form a positively charged sulfonium ion and cleavage to THT, which is further oxidized to secondary metabolites.

liver [1,12]. As shown in Fig. 1 busulfan is conjugated with glutathione via a two step mechanism primarily mediated by glutathione *S*-transferase A1-1 (GST A1-1) [13,14]. The intermediate product is a positively charged sulfonium ion which can be cleaved to tetrahydrothiophene (THT) either enzymatically for example by β-lyases or chemically by alkalization. Quantitative determination of THT is assumed to accurately reflect the initial step of busulfan biotransformation. Since in vitro experiments are useful for investigating busulfan biotransformation, which may be a modulator of concentration and hence side effects such as VOD, a sensitive method for THT determination is required. Several GC-methods are published so far using flame ionisation detection (FID) [15] or mass selective (MS) detection [14,16,17]. However, they are either rather insensitive (lower limit of detection 1–5 $\mu g\,ml^{-1}$) [15,16] or lack a proper validation procedure [14,17]. We therefore developed an improved GC–MS method for the sensitive determination of THT. The method is applied to carry out enzyme kinetic studies as well as to assess GST A1-1 activity in human liver cytosol.

2. Experimental

2.1. Chemicals

All solvents used were of HPLC quality; chemicals were of analytical grade. Tetrahydrothiophene, 2-ethylthiophene, busulfan and glutathione were obtained from Sigma (Deisenhofen, Germany), sodium dihydrogen phosphate monohydrate and EDTA were supplied by Merck (Darmstadt, Germany).

2.2. Preparation of human liver cytosol

Human liver cytosol was prepared from a sample obtained as a surgical waste during partial hepatectomy. Crude homogenate was centrifuged at 10 000 *g* for 5 min followed by a centrifugation step at 100 000 *g* [18]. The cytosolic 100 000 *g* supernatant

was used in the following experiments. Protein contents were determined according to the method of Lowry et al. [19].

2.3. Incubation of human liver cytosol with busulfan

Incubation mixtures contained 1.0 mg cytosolic protein and 0.7 μmol glutathione in 1.0 ml of assay buffer (100 m*M* sodium phosphate, pH 7.4; 1 m*M* EDTA). Preincubations were carried out for 5 min at 37°C followed by addition of busulfan in DMSO and incubation for 20 min at 37°C. The enzymatic reactions were stopped by adding 5 ml of dichloromethane and after vortexing the mixture was centrifuged for 15 min at 450 *g*. For alkaline hydrolysis and THT extraction 250 μl *n*-pentane, 15 μl 2-ethylthiophene (10 μg ml^{-1}; internal standard) and 50 μl NaOH (1 *M*) were added. After another vortexing step and centrifugation for 30 min at 1800 *g* samples were stored at −20°C and the *n*-pentane phase was removed from the frozen samples for analysis.

2.4. Incubation of recombinant GSTs

Various amounts (10 and 25 μg) of the purified recombinant glutathione *S*-transferases A1-1, M1-1 and P1-1 (Calbiochem-Novabiochem, La Jolla, CA, USA) were added to the incubation mixture containing 1 mg of heat-inactivated cytosolic protein and were treated in the same way as cytosolic incubations.

2.5. Calibration and standardization

Stock solutions of busulfan were prepared in DMSO at 100 mM; stock standard solutions of THT and 2-ethylthiophene were each prepared in a 1:1 mixture of isopropanol and water at 1.0 mg ml^{-1} and stored at −20°C. Working standard solutions were freshly prepared from these stock solutions. Calibration samples were prepared by diluting the THT standard solution in assay buffer containing heat-inactivated liver cytosol (1 mg protein ml^{-1}). The final THT concentrations were 10.0, 50.0, 100.0, 250.0, 500.0 or 1000.0 ng ml^{-1}, respectively. Internal standard, 1 M-NaOH and *n*-pentane were added and the mixture was treated as described above. Calibration curves were obtained by plotting the peak area ratio of THT and the internal standard 2-ethylthiophene against the substance concentration added.

2.6. Instrumentation and GC conditions

The GC–MS-system consisted of a HP 5890A II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) with a TSQ 700 mass-selective detector (Finnigan MAT, Bremen, Germany). GC was performed in the splitless mode on a 25 m×0.2 mm HP 5-MS capillary column (methylpolysiloxane with 5% phenyl groups), film thickness 0.33 μm (J&W Scientific, Fisons, Mainz, Germany), to which helium was supplied as carrier gas using the constant flow mode with an inlet pressure of 100 kPa. An aliquot (2 μl) of *n*-pentane was injected at 280°C with an autosampler A200S (CTC Analytics, Zwingen, Switzerland) cooled at 4°C. The initial oven temperature of 45°C was held for 1.5 min, increased in a first step by 20°C min^{-1} to 100°C and in a second step by 35°C min^{-1} to 300°C. THT and 2-ethylthiophene eluted at 3.4 and 4.1 min, respectively.

MS was performed in the electron impact (EI) mode. MS conditions were: transfer-line temperature 300°C, source temperature 150°C, emission current 350 μA, electron energy 70 eV, multiplier voltage 1600 V. Measurements were carried out in the selective ion monitoring (SIM) mode and data acquisition was performed with ICIS software, Finnigan MAT (Bremen, Germany).

2.7. Assay validation

For determination of assay accuracy and variability various amounts of THT were added to 1 ml of mixtures (final concentrations 10, 25, 250 and 1000 ng ml^{-1}) containing heat-inactivated liver cytosol (1 mg protein). These mixtures were then analyzed alone (accuracy) or together with the incubation samples in every series of experiments as quality controls (variability).

3. Results and discussion

The method described allows the sensitive determination of THT after conjugation of busulfan with glutathione by human liver GST A1-1 and is based on combination of high-resolution GC with highly selective mass spectrometry. After gas chromatography of incubation samples mass spectra were scanned and the fragment ion with the highest intensity was chosen to be determined in the SIM mode, in order to achieve maximal sensitivity. Authenticity of THT in cytosolic incubation mixtures was proven by comparing the scanned mass spectra and retention times of incubation samples and an authentic reference compound. Scanned mass spectra showed the following main fragments with their intensities: THT: M+ 88(100); *m/z* 87(30); *m/z* 60(95); *m/z* 54(17); and 2-ethylthiophene: M+ 112(43); *m/z* 111(9); *m/z* 97(100); *m/z* 77(4). Analytes were measured in the SIM mode at *m/z* 88 and 97 with retention times of 3.4 and 4.1 for THT and the internal standard 2-ethylthiophene, respectively (Fig. 2). The resulting chromatograms of an incubation blank, a calibration sample, an incubation sample with the corresponding heat-inactivated sample and a sample with enzyme inhibition are depicted in Fig. 2. A nearly total inhibition of busulfan transformation can be observed after adding 1 μmol of the glutathione *S*-transferase inhibitor ethacrynic acid to the incubation mixtures. Standardization was carried out using heat-inactivated human liver cytosol instead of the native cytosols. The method showed good linearity over the entire concentration range of 5–1000 ng ml^{-1}. Even at the lowest concentration the signal-to-noise ratio was better than 5. A typical standard curve for the determination of

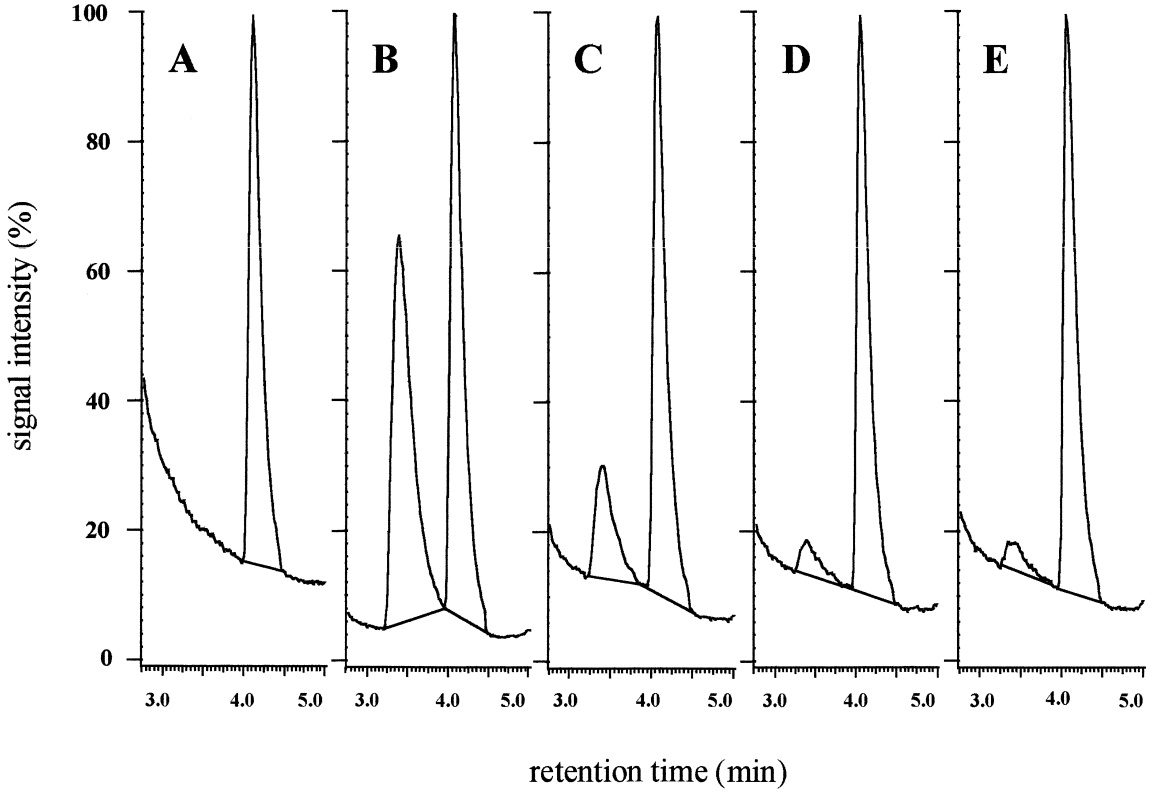

Fig. 2. Chromatograms of mixtures incubated for 20 min at 37°C in the presence of 1 mg ml^{-1} protein and 150 ng ml^{-1} 2-ethylthiophene as internal standard. (A) Incubation blank containing heat-inactivated human liver cytosol. (B) Calibration sample containing 100 ng ml^{-1} THT and heat-inactivated liver cytosol. (C) Liver cytosol after incubation with 0.6 m*M* busulfan. (D) Heat-inactivated liver cytosol after incubation with 0.6 m*M* busulfan. (E) Liver cytosol after preincubation with 1 m*M* ethacrynic acid.

Table 1
Accuracy of the determination of THT in incubation mixtures containing heat-inactivated human liver cytosol

Concentration added (ng ml^{-1})	Concentration found (ng ml^{-1})	Recovery (%)
10.0	9.9	98.6
50.0	48.0	96.0
100.0	96.9	96.9
250.0	258.9	103.6
500.0	492.8	98.6
1000.0	895.3	89.5

THT was $y=0.0035x+0.0108$ ($r>0.9997$). There was a good correlation ($y=0.90x+13.95$, $r=0.9983$) between the concentration added and that measured by GC–MS as indicated by the values for accuracy (Table 1). Reproducibility was tested by repeated analyses of mixtures of inactivated liver cytosols spiked with known amounts of THT. Table 2 shows the intra-assay and the inter-assay variabilities. Inter-assay variability was better than 6% except the lowest concentration (10 ng ml^{-1}), which increased to approximately 13%. For 5 ng ml^{-1} the coefficient of variation in the intra-assay data was approximately 20% with an acceptable bias of 8%. Therefore, the limit of quantification of THT was set at 5 ng ml^{-1} and the limit of detection (signal-to-noise ratio>3) was 2 ng ml^{-1}.

The suitability of the method described has been tested during an enzyme kinetic study. Busulfan was incubated with human liver cytosol in the concentration range from 62.5 μ*M* to 5 m*M* and the concentration of THT was determined. Preliminary experiments showed that THT formation was linear for protein contents from 200 μg to 1 mg, for glutathione concentrations up to 1 m*M* and for incubation time up to 30 min. In addition, the difference between enzymatic and spontaneous THT formation was maximal at pH 7.4. Therefore incubations were carried out for 20 min at 37°C and pH 7.4 in the presence of 1 mg cytosolic protein and 0.7 μmol glutathione. Since solubility of busulfan in aqueous media is poor we used dimethylsulfoxide (DMSO) as solvent with a stock concentration at 250 m*M* thereby obtaining a maximal final concentration of 5 m*M*. The resulting data were fitted with a Michaelis-Menten kinetic as shown in Fig. 3. The maximum rate of THT formation (V_{max}) was calculated as 133.7 pmol mg^{-1} min^{-1} with a K_m value of 2.74 m*M*. Gibbs et al. [17] described THT formation rate as a linear function of busulfan concentration. However, the maximum busulfan concentration was 1200 μ*M* limited by the solubility of the drug in aqueous media. Using dimethylsulfoxide instead of acetonitrile as solvent for busulfan we obtained higher busulfan concentrations in the incubation mixture. Thus, the enzyme was almost saturated which enabled us to calculate the enzyme kinetic parameters of THT formation as a result of busulfan conjugation. This approach allows to estimate V_{max} and K_m, from which the intrinsic clearance can be calculated. Furthermore, incubations with recombinant glutathione *S*-transferases were carried out to

Table 2
Intra- and inter-assay variability in the determination of tetrahydrothiophene

Concentration added (ng ml^{-1})	*n*	Concentration found (ng ml^{-1})	Recovery (%)	Bias (%)	C.V. (%)
Intra-assay variability					
5.0	6	4.61±0.90	92.2	−7.8	19.5
10.0	6	10.6±0.99	106.0	6.0	9.4
25.0	6	26.5±1.47	105.8	5.8	5.5
250.0	6	250.6±7.97	100.2	0.2	3.2
1000.0	6	993.0±34.6	99.3	−0.7	3.5
Inter-assay variability					
10.0	12	10.9±1.37	108.9	8.9	12.6
25.0	12	25.1±1.02	100.4	0.4	4.1
250.0	12	247.8±15.3	99.1	−0.9	6.2
1000.0	12	1001.0±43.9	100.1	0.1	4.4

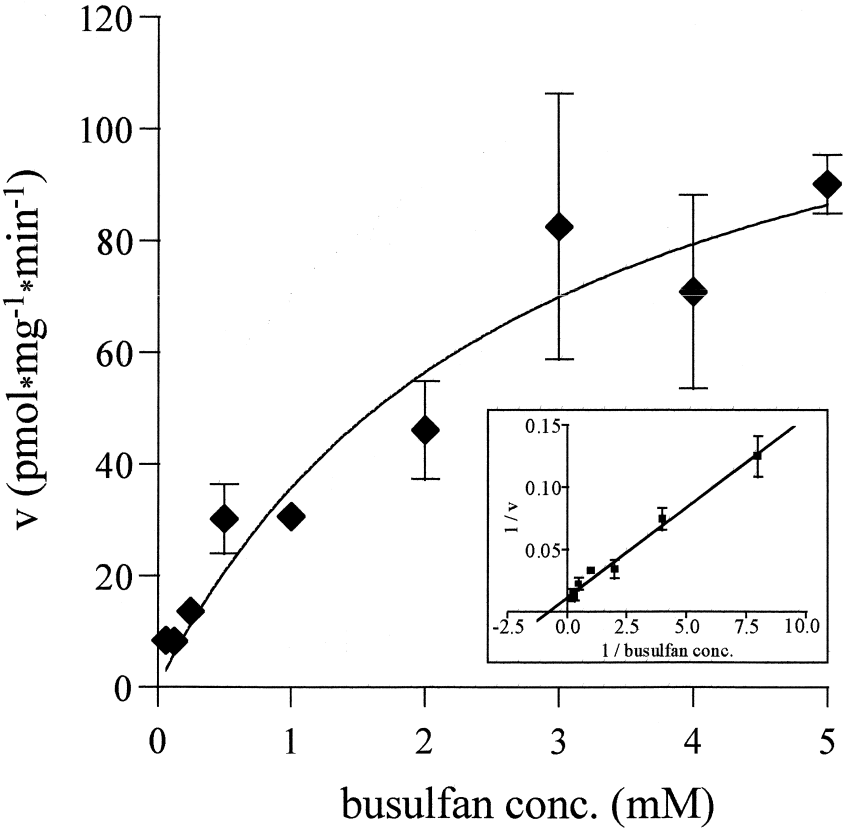

Fig. 3. Kinetic of THT formation after incubation of busulfan with human liver cytosol. Mixtures contained 1 mg protein and were incubated with different concentrations of busulfan at 37°C for 20 min. Values represent mean±SD (n=3).

investigate the affinity of busulfan towards different enzymes of this family. Conditions of incubation were the same as in cytosolic incubations containing 10 and 25 $\mu g\,ml^{-1}$ of recombinant protein. Compared to Czerwinski et al. [14] our determination of busulfan metabolism resulted in similar findings. As shown in Fig. 4 transformation to THT in incubations with glutathione *S*-transferase A1-1 is 6-fold compared to the non-enzymatic reaction, incubations with the isoform P1-1 results in about 13% of the A1-1 amount and even at high protein concentrations there is almost no difference in incubations with M1-1 compared to control values. In contrast to this Czerwinski et al. found remarkable activity of glutathione *S*-transferase M1-1 in their incubation. However, Czerwinski et al. used purified enzymes from human liver tissues standardized on the activity towards CDNB, a non-specific substrate. In contrast, our findings were based on the use of equal amounts of highly purified recombinant proteins.

In summary our data demonstrate that the GC–MS method described is suitable for sensitive quantification of tetrahydrothiophene after incubation of busulfan with cytosolic protein. This validated assay may be a valuable tool for further biotransformation studies of busulfan in different tissues, in particular since busulfan seems to be a specific substrate for the glutathione *S*-transferase isoenzyme A1-1.

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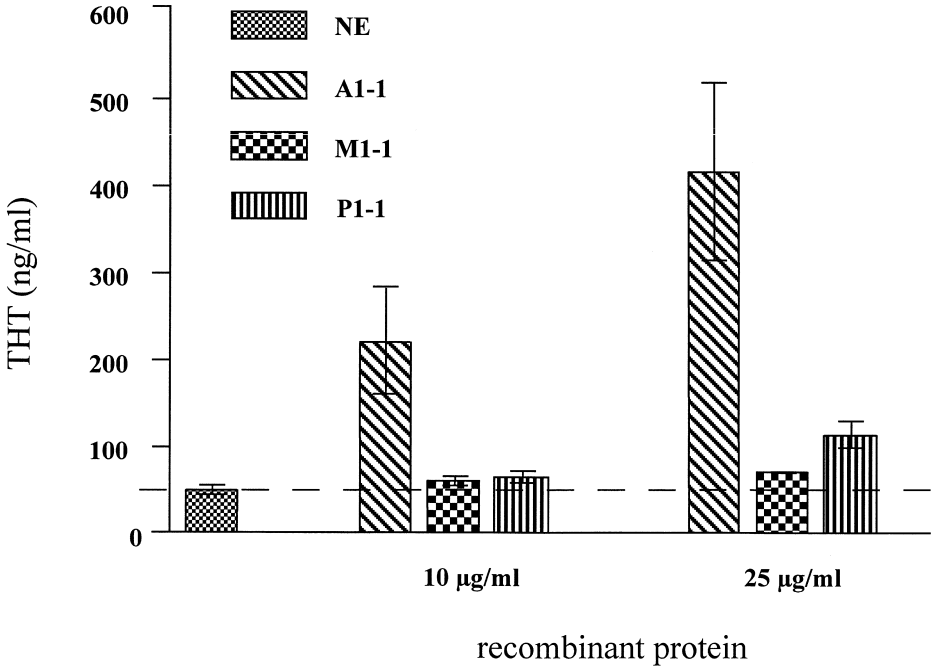

Fig. 4. THT formation after incubation of busulfan with recombinant protein of glutathione *S*-transferase isoenzymes A1-1, M1-1 and P1-1 and heat-inactivated liver cytosol as a negative control for non-enzymatic reaction (NE). Mixtures contained 0.6 m*M* busulfan which were incubated at 37°C for 20 min with 10 and 25 $\mu g\,ml^{-1}$ recombinant protein, respectively. Values represent mean±min/max ($n=2$).

References

[1] M. Hassan, P. Ljungman, P. Bolme, O. Ringden, Z. Syruckova, A. Bekassy, J. Stary, I. Wallin, N. Kallberg, Blood 84 (1994) 2144.
[2] M.B. Regazzi, F. Locatelli, I. Buggia, F. Bonetti, M. Zecca, M. Pregnolato, S. Quaglini, Clin. Pharmacol. Ther. 54 (1993) 45.
[3] G. Vassal, A. Fischer, D. Challine, I. Boland, F. Ledheist, S. Lemerle, E. Vilmer, C. Rahimy, G. Souillet, E. Gluckman, G. Michel, A. Deroussent, A. Gouyette, Blood 82 (1993) 1030.
[4] M. Hassan, G. Oberg, H. Ehrsson, M. Ehrnebo, I. Wallin, B. Smedmyr, T. Totterman, S. Eksborg, B. Simonsson, Eur. J. Clin. Pharmacol. 36 (1989) 525.
[5] S.I. Bearman, Blood 85 (1995) 3005.
[6] H.M. Shulman, W. Hinterberger, Bone Marrow Transplant 10 (1992) 197.
[7] G. Vassal, S. Koscielny, D. Challine, D. ValteauCouanet, I. Boland, A. Deroussent, J. Lemerle, A. Gouyette, O. Hartmann, Cancer Chemother. Pharmacol. 37 (1996) 247.
[8] L.B. Grochow, R.J. Jones, R.B. Brundrett, H.G. Braine, T.L. Chen, R. Saral, G.W. Santos, O.M. Colvin, Cancer Chemother. Pharmacol. 25 (1989) 55.
[9] S.P. Dix, J.R. Wingard, R.E. Mullins, I. Jerkunica, T.G. Davidson, C.E. Gilmore, R.C. York, L.S. Lin, S.M. Devine, R.B. Geller, L.T. Heffner, C.D. Hillyer, H.K. Holland, E.F. Winton, R. Saral, Bone Marrow Transplant. 17 (1996) 225.
[10] P.J. Shaw, C.E. Scharping, R.J. Brian, J.W. Earl, Blood 84 (1994) 2357.
[11] M. Hassan, G. Oberg, M. Bjorkholm, I. Wallin, M. Lindgren, Cancer Chemother. Pharmacol. 33 (1993) 181.
[12] J.J. Roberts, G.P. Warwick, Biochem. Pharmacol. 6 (1961) 217.
[13] M. Hassan, H. Ehrsson, Drug Metab. Dispos. 15 (1987) 399.
[14] M. Czerwinski, J. Gibbs, J.T. Slattery, Drug Metab. Dispos. 24 (1996) 1015.
[15] W. Onkenhout, W.M.G.M. van Loon, W. Buijs, A. van der Gen, N.P.E. Vermeulen, Drug Metab. Dispos. 14 (1986) 608.
[16] D.H. Merchand, M.M. Abdel-Monem, Biochem. Biophys. Res. Commun. 128 (1985) 360.
[17] J.P. Gibbs, M. Czerwinski, J.T. Slattery, Cancer Res. 56 (1996) 3678.
[18] P.J. Meier, H.K. Müller, B. Dick, U.A. Meyer, Gastroenterology 85 (1983) 682.
[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.