Journal of Chromatography, 612 (1993) 223–230
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6647

Achiral and chiral high-performance liquid chromatographic determination of tramadol and its major metabolites in urine after oral administration of racemic tramadol

B. Elsing and G. Blaschke

Department of Pharmaceutical Chemistry, University of Münster, W-4400 Münster (Germany)

(First received August 28th, 1992; revised manuscript received October 26th, 1992)

ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the simultaneous determination of tramadol and its major metabolites O-demethyltramadol and N-demethyltramadol in urine has been developed. The determination of the enantiomeric ratios of the three compounds was achieved using a Chiralpak AD column and a Chiralcel OD column, respectively. After oral administration of racemic tramadol to five healthy volunteers, inter-individual differences of the excreted amounts and the enantiomeric ratios of the compounds were observed.

INTRODUCTION

Tramadol hydrochloride (*rac*-1-(*e*)-(*m*-methoxyphenyl)-2-(*e*)-(dimethylaminomethyl)cyclohexan-1-(*a*)-ol hydrochloride, Tramal) is a centrally acting analgesic drug that is used in therapy as the racemate [1]. The major phase I metabolites of tramadol (**1**, Fig. 1) in humans are the pharmacologically active O-demethyltramadol (**2**) and the inactive N-demethyltramadol (**3**) [2]. The resolution of the enantiomers of tramadol on a preparative scale can be achieved by fractional crystallization of the dibenzoyltartrate [1] or the mandelate [3].

The enantiomers of tramadol differ in their pharmacological activities. The (+)-enantiomer exhibited a ten-fold higher analgesic activity than the (−)-enantiomer [1]. To date, no information is available on the stereoselective biotransformation of tramadol in humans.

This paper describes the determination of tramadol and its main metabolites **2** and **3** by high-performance liquid chromatography (HPLC) on a reversed-phase column. Furthermore, the enantiomeric ratios of **1**, **2** and **3** in humans were analysed by chiral HPLC. The applicability of these assays is demonstrated by the quantification and the enantioseparations of the three compounds from human urine after oral administration of racemic tramadol hydrochloride to five healthy volunteers.

EXPERIMENTAL

Chemicals

Tramadol hydrochloride was a gift from Grünenthal (Stolberg, Germany). O-Demethyltra-

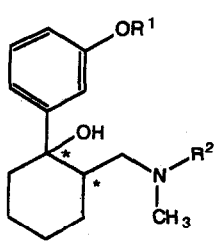
Correspondence to: G. Blaschke, Department of Pharmaceutical Chemistry University of Münster, W-4400, Münster, Germany.

0378-4347/93/$06.00

madol (2), N-demethyltramadol (3), N,O-didemethyltramadol (4) and the ethoxy analogue of tramadol (used as the internal standard, I.S.) were synthesized according to the literature [4] and characterized by their spectral data. Ethyl acetate, *n*-hexane and 2-propanol were LiChrosolv reagents (Merck, Darmstadt, Germany). The other chemicals were obtained from commercial sources and were of analytical grade. Buffer solutions were prepared in doubly distilled, deionized water. Both enantiomers of tramadol (enantiomeric excess > 99%) were obtained by fractional crystallization of the racemate as salts of (+)- and (−)-mandelic acid, respectively, using *n*-hexane–ethanol (4:1, v/v) [3].

Apparatus

The chromatographic system consisted of a L-6000 liquid chromatograph (Merck-Hitachi), a Rheodyne sample injector (Model 7125, Rheodyne) equipped with a 50-μl loop, a 655 A variable-wavelength detector (Merck-Hitachi) set at 270 nm, and a D-2000 chromato-integrator (Merck-Hitachi).



R^1	R^2	
CH_3	CH_3	tramadol (1)
H	CH_3	O-demethyltramadol (2)
CH_3	H	N-demethyltramadol (3)
H	H	N,O-didemethyltramadol (4)
C_2H_5	CH_3	ethoxy analogue (I.S.)

Fig. 1. Structures of tramadol (1), O-demethyltramadol (2), N-demethyltramadol (3), N,O-didemethyltramadol (4) and the ethoxy analogue of tramadol (I.S.). The chiral centres are marked with asterisks.

Achiral chromatography

The analytical separation was obtained on an RP Select B column (Merck, 5 μm particle size, 250 mm × 4.0 mm I.D.) equipped with an RP Select B guard column (5 μm particle size, 25 mm × 4.0 mm I.D.). The mobile phase was 0.05 *M* phosphate buffer (pH 3.0)–methanol (70:30, v/v). The flow-rate was 1.0 ml/min.

Chiral chromatography

The enantioseparations of tramadol and N-demethyltramadol were achieved on an amylose tris-3,5-dimethylphenyl carbamate chiral stationary phase (Chiralpak AD, Daicel, Tokyo, Japan, 10 μm particle size, 250 mm × 4.6 mm I.D.) with a Chiralpak AD guard column (10 μm particle size, 25 mm × 4.6 mm I.D.). Two different mobile phase compositions were used. Mobile phase A was *n*-hexane–2-propanol–diethylamine (97.5:2.5:0.01, v/v), flow-rate 1.0 ml/min. Mobile phase B was *n*-hexane–ethanol–diethylamine (94.0:6.0:0.01, v/v), flow-rate 1.2 ml/min. O-Demethyltramadol was resolved on a cellulose tris-3,5-dimethylphenyl carbamate column (Chiralcel OD, Daicel, 10 μm particle size, 250 mm × 4.6 mm I.D.) equipped with a Chiralcel OD guard column (10 μm particle size, 25 mm × 4.6 mm I.D.). The mobile phase was *n*-hexane–ethanol–water (96.0:4.0:0.1, v/v). The flow-rate was 0.7 ml/min.

Urine collection

Five healthy volunteers took part in the study. The administered dose was 100 mg of tramadol hydrochloride (Tramal). Urine was collected during the following intervals: 0–1, 1–2, 2–4, 4–6, 6–8, 8–10, 10–12, 12–14, 14–24 and 24–30 h. The volume and pH of the urine were recorded. Urine samples were stored frozen at −20°C until analysis.

Extraction of urine samples

For the achiral simultaneous determination of tramadol, O-demethyltramadol and N-demethyltramadol, 1.0 ml of urine was adjusted to *ca.* pH 9 by the addition of 50–100 μl of 0.05 *M* Tris buffer (pH 10.5), and 50 μl of a solution of the

I.S., the ethoxy analogue of tramadol (50 μg/ml in water), were added to each urine sample. The samples were extracted twice with 4.0 ml of *n*-hexane–ethyl acetate (80:20, v/v) by shaking for 15 min. The organic layers were separated by centrifugation (15 min at 3000 *g*), combined and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase, and 50 μl were injected into the HPLC system.

For the determination of the enantiomeric ratios of tramadol, O-demethyltramadol and N-demethyltramadol the extraction was modified. Urine samples of 1.0 ml were adjusted to a pH > 10 by the addition of aqueous ammonia (25%). Tramadol and its N-demethylated metabolite **3** were extracted with 4.0 ml of *n*-hexane. The organic phase was separated by centrifugation, removed and evaporated under a stream of nitrogen. The phenolic metabolite **2** was extracted in a second step with 4.0 ml of *n*-hexane–ethyl acetate (80:20, v/v) after the addition of 0.1 *M* HCl to adjust the pH to 9. The organic layer was removed after centrifugation and evaporated under a stream of nitrogen. The residues were dissolved in 100 μl of the appropriate mobile phase, and 50 μl were analysed on Chiralpak AD and Chiralcel OD, respectively.

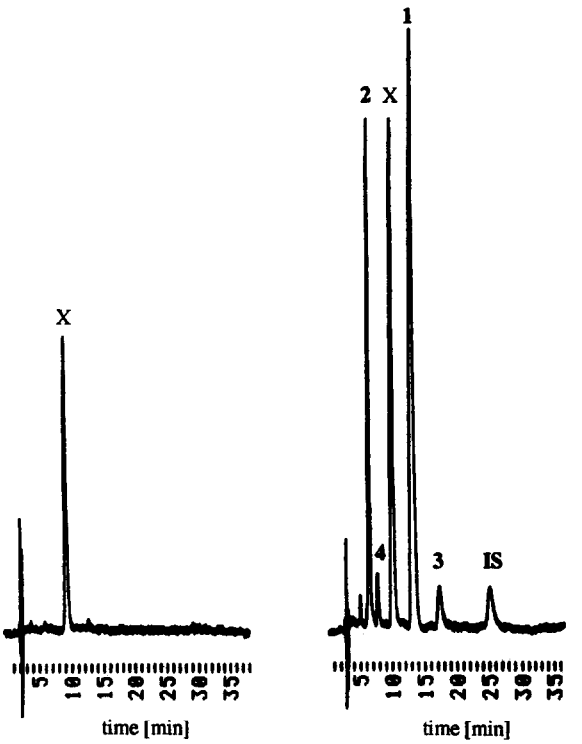


Fig. 2. Chromatograms of (left) blank urine and (right) tramadol (**1**), O-demethyltramadol (**2**), N-demethyltramadol (**3**), N,O-didemethyltramadol (**4**) and the ethoxy analogue as internal standard (I.S.) extracted from human urine after oral administration of tramadol hydrochloride (volunteer E, urine collection 2–4 h after administration). Peak X = unknown constituent of urine. Chromatographic conditions: column, RP Select B; mobile phase, 0.05 *M* phosphate buffer (pH 3.0)–methanol (70:30, v/v); flow-rate, 1.0 ml/min; detection wavelength, 270 nm.

RESULTS AND DISCUSSION

Quantitative analysis

The separation of tramadol (**1**), its major metabolites O-demethyltramadol (**2**) and N-demethyltramadol (**3**) and the ethoxy analogue of tramadol (I.S.) was achieved on a reversed-phase column. Fig. 2 shows representative chromatograms of blank urine and urine sampled 4 h after oral administration of tramadol hydrochloride. The retention times for **2**, **1**, **3** and the I.S. were 5.9, 12.8, 16.8 and 24.9 min, respectively. All compounds were resolved from interfering peaks of the urine.

The concentrations of **1**, **2** and **3** were calculated from calibration curves obtained by the analysis of urine samples spiked with known amounts of the I.S., **1**, **2** and **3** (Table I). Multiple repetition of the calibration experiments ensured the precision and reproducibility of the assay. The mean recovery (± S.E.M.) of tramadol was 69.3 ± 3.1%, that of O-demethyltramadol 65.5 ± 3.6% and that of N-demethyltramadol 68.1 ± 2.8%. The detection limit was *ca.* 80 ng/ml urine for all three compounds.

Cumulative excretion curves

The cumulative excretion curves of tramadol, O-demethyltramadol and N-demethyltramadol in urine after oral administration of 100 mg of tramadol hydrochloride (equivalent to 87.85 mg of **1**) to a healthy volunteer are shown in Fig. 3. In agreement with previous studies, inter-individ-

TABLE I

DETERMINATION OF TRAMADOL, O-DEMETHYLTRAMADOL AND N-DEMETHYLTRAMADOL

Assay precision calculated from spiked urine samples.

Tramadol (1)			O-Demethyltramadol (2)			N-Demethyltramadol (3)		
Amount added (μg/ml)	*n*	Peak-area ratio (mean ± S.E.M.) (area 1/area I.S.)	Amount added (μg/ml)	*n*	Peak-area ratio (mean ± S.E.M.) (area 2/area I.S.)	Amount added (μg/ml)	*n*	Peak-area ratio (mean ± S.E.M.) (area 3/area I.S.)
1.64	3	1.04 ± 0.02	2.19	5	1.75 ± 0.14	1.10	3	0.47 ± 0.02
2.20	5	1.58 ± 0.07	3.18	3	3.06 ± 1.03	2.02	5	1.17 ± 0.05
3.27	4	2.05 ± 0.16	4.37	3	4.91 ± 0.96	2.19	4	1.42 ± 0.13
4.40	3	3.02 ± 0.24	6.36	3	5.32 ± 0.39	4.37	3	2.95 ± 0.55
8.80	4	5.99 ± 0.64	8.74	4	8.01 ± 0.18	8.74	4	5.56 ± 0.54
17.60	4	10.55 ± 0.55	17.48	4	12.81 ± 0.08	17.48	4	10.06 ± 0.46
35.20	4	21.53 ± 0.55	34.96	4	25.99 ± 0.15			

ual differences of the excreted amounts of the three compounds were found [2]. The cumulative excretion of **1**, **2** and **3** ranged from 25 to 52% of the administered dose, which may be explained by different urinary flow-rates [5] and/or urinary pH values [6]. The pH of the urine of the volunteers was not standardized by means of oral administration of ammonium chloride to achieve a controlled pH-dependent elimination of the basic drug. Four of five volunteers exhibited similar metabolic behaviour, as demonstrated for volunteer A (Fig. 3). Differences concerning the eliminated amounts of O-demethyltramadol (**2**) were determined for volunteers A and B and volunteers C and D (Table II). The fifth subject excreted a significantly larger amount of N-demethyltramadol (**3**) and very little of the phenolic metabolite **2** (Table II, volunteer E).

These results may be explained by an individual genetic disposition to express specific cytochrome P450 isoenzymes. The demethylation of a variety of compounds, including drugs structurally related to **1**, is catalysed by specific cytochrome P450 isoenzymes [7–9]. *In vitro* incubation of tramadol with liver microsomes in the presence of the cytochrome P450 IID6 isoenzyme inhibitors sparteine and quinidine indicated that this isoenzyme might at least partially catalyse the O-demethylation of **1** [10]. However, further experiments with specific antibodies against P450 isoenzymes [11] will be necessary to prove that cytochrome P450 IID6 isoenzyme mediates the O-demethylation of tramadol.

Determination of enantiomeric ratios

The enantioseparation of tramadol and its N-demethylated metabolite **3** was achieved by HPLC on a Chiralpak AD column (Fig. 4a), and

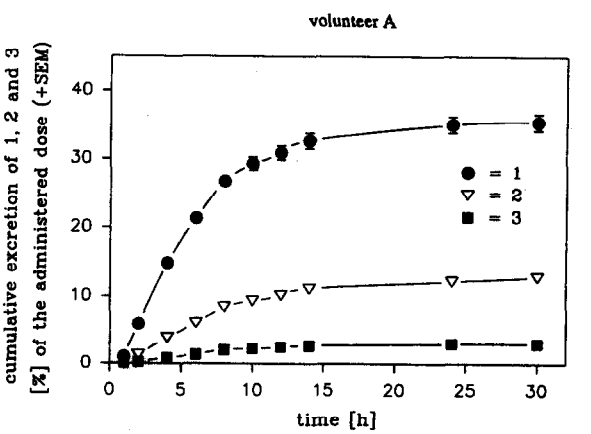


Fig. 3. Cumulative excretion curves of (●) tramadol (1), (▽) O-demethyltramadol (2) and (■) N-demethyltramadol (3) of a volunteer after oral administration of 100 mg of tramadol hydrochloride.

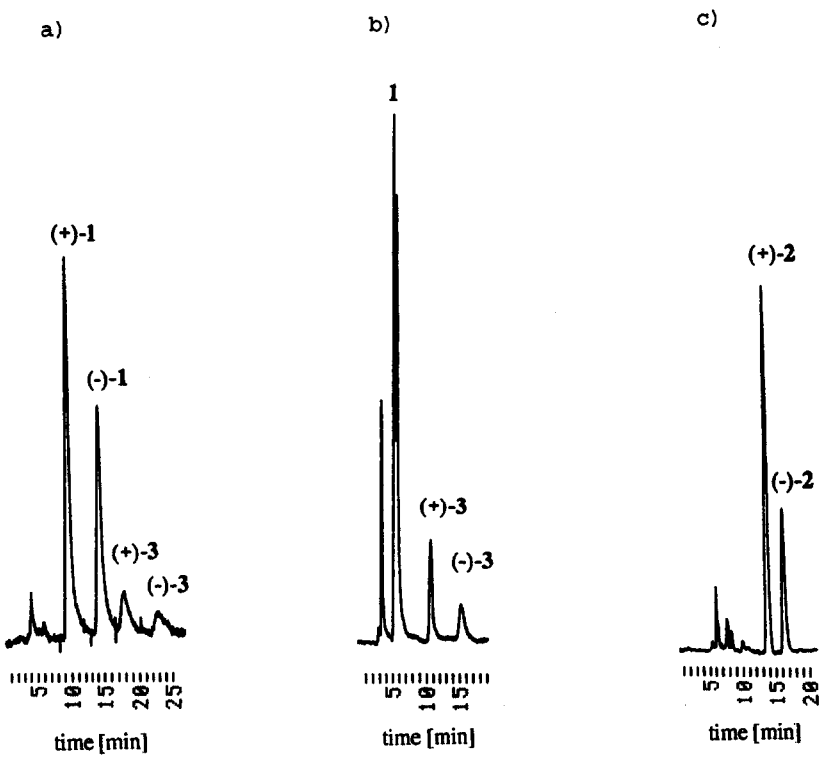


Fig. 4. Chromatograms showing the enantioseparations of **1**, **2** and **3** extracted from human urine after oral administration of 100 mg of racemic tramadol hydrochloride. (a) Enantioseparation of **1** and **3** (volunteer E, urine collection 2–4 h after administration). Chromatographic conditions: column, Chiralpak AD; mobile phase, *n*-hexane–2-propanol–diethylamine (97.5:2.5:0.01, v/v); flow-rate, 1.0 ml/min; detection wavelength, 270 nm. (b) Enantioseparation of **3** (volunteer E, urine collection 8–10 h after administration); peak 1 = unresolved enantiomers of tramadol. Chromatographic conditions: column, Chiralpak AD; mobile phase, *n*-hexane–ethanol–diethylamine (94.0:6.0:0.01, v/v); flow-rate, 1.2 ml/min; detection wavelength, 270 nm. (c) Enantioseparation of **2** (volunteer B, urine collection 14–24 h after administration). Chromatographic conditions: column, Chiralcel OD; mobile phase, *n*-hexane–ethanol–water (96.0:4.0:0.1, v/v); flow-rate, 0.7 ml/min; detection wavelength, 270 nm.

TABLE II

DISTRIBUTION OF TRAMADOL, O-DEMETHYLTRAMADOL AND N-DEMETHYLTRAMADOL

Values were calculated as percentages of the cumulative excreted amounts up to 30 h after oral administration of 100 mg of tramadol hydrochloride.

Volunteer	Cumulative excretion (mg)	Tramadol (%)	O-Demethyl-tramadol (%)	N-demethyl-tramadol (%)
A	45.00	63.23	25.09	5.68
B	26.80	69.16	25.92	4.92
C	22.63	54.00	43.57	2.43
D	22.02	55.14	43.74	1.12
E	45.64	78.59	8.69	12.72

TABLE III

ENANTIOMERIC RATIO OF TRAMADOL IN HUMAN URINE AFTER ORAL ADMINISTRATION OF 100 mg OF RACEMIC TRAMADOL HYDROCHLORIDE

Time (h)	Enantiomeric ratio of tramadol (+/−)				
	Volunteer A	Volunteer B	Volunteer C	Volunteer D	Volunteer E
0–1	1.09	1.04	1.08	1.00	1.01
1–2	1.11	1.07	0.99	1.03	1.04
2–4	1.14	1.06	0.96	1.11	1.16
4–6	1.16	1.29	0.98	0.99	1.26
6–8	1.17	1.42	0.99	1.01	1.31
8–10	1.18	1.46	1.04	1.06	1.33
10–12	1.22	1.50	1.09	1.04	1.34
12–14	1.22	1.57	1.05	1.03	1.47
14–24	1.29	1.81	1.05	1.14	1.43

the enantiomers of O-demethyltramadol (**2**) were resolved on a Chiralcel OD column (Fig. 4c). The enantiomers of the phenolic metabolite **2** were not resolved on the Chiralpak AD column but interfered with N-demethyltramadol (**3**). Owing to the incompatibility of an aqueous mobile phase with Chiralpak AD and Chiralcel OD, column-switching techniques could not be applied. Therefore, **1** and **3** were extracted from urine at pH > 10 prior to the extraction of **2** at pH 9. Above pH 10 the phenolic metabolite **2** remained in the aqueous phase.

Tramadol and N-demethyltramadol could be separated simultaneously using mobile phase A (Fig. 4a). The k' and α values for the enantiomers of **1** and N-demethyltramadol (**3**) were $k'_1 = 1.94$, $k'_2 = 2.86$, $\alpha = 1.91$ and $k'_1 = 3.73$, $k'_2 = 5.14$, $\alpha = 1.38$, respectively. However, in urine samples with high concentrations of **1**, the (−)-enantiomer of **1** interfered with the peak of the

TABLE IV

ENANTIOMERIC RATIO OF N-DEMETHYLTRAMADOL IN HUMAN URINE AFTER ORAL ADMINISTRATION OF 100 mg OF RACEMIC TRAMADOL HYDROCHLORIDE

Time (h)	Enantiomeric ratio of N-demethyltramadol (+/−)				
	Volunteer A	Volunteer B	Volunteer C	Volunteer D	Volunteer E
0–1	2.63	2.71	2.39	−[a]	1.54
1–2	2.00	1.83	2.68	−[a]	1.23
2–4	4.00	1.84	1.52	1.59	1.34
4–6	3.25	2.82	2.40	−[a]	1.66
6–8	3.34	3.68	2.51	−[a]	1.57
8–10	4.58	3.95	−[a]	2.50	1.56
10–12	4.78	6.13	−[a]	3.61	1.69
12–14	3.84	6.14	−[a]	−[a]	1.64
14–24	5.37	6.55	−[a]	−[a]	2.08

[a] Not determined.

TABLE V

ENANTIOMERIC RATIO OF O-DEMETHYLTRAMADOL IN HUMAN URINE AFTER ORAL ADMINISTRATION OF 100 mg OF RACEMIC TRAMADOL HYDROCHLORIDE

Time (h)	Enantiomeric ratio of O-demethyltramadol (+/−)				
	Volunteer A	Volunteer B	Volunteer C	Volunteer D	Volunteer E
0–1	1.05	1.11	1.83	1.54	0.46
1–2	1.06	1.12	1.78	1.73	0.17
2–4	1.13	1.23	1.76	1.83	0.23
4–6	1.24	1.39	1.85	1.78	0.25
6–8	1.33	1.56	1.93	1.84	0.28
8–10	1.51	1.71	2.05	1.96	0.30
10–12	1.51	1.90	1.68	1.88	0.27
12–14	1.81	2.15	1.92	2.11	0.35
14–24	1.83	2.28	1.72	2.00	0.33

(+)-enantiomer of **3**. A better resolution of the enantiomers of **3** could be achieved by substituting ethanol for 2-propanol in the mobile phase (Fig. 4b). The enantiomers of tramadol were not resolved under these conditions. The k' and α values for the enantiomers of **3** were $k'_1 = 2.45$, $k'_2 = 4.17$, $\alpha = 1.70$.

The enantiomers of **2** were separated on a Chiralcel OD column with k' and α values of $k'_1 = 2.49$, $k'_2 = 3.16$, $\alpha = 1.27$ (Fig. 4c). The (+)-enantiomers of all three compounds eluted first from Chiralpak AD and Chiralcel OD, respectively [10].

The enantiomeric ratios of **1**, **2** and **3** were determined in human urine after oral administration of 100 mg of racemic tramadol hydrochloride to five healthy volunteers (Tables III–V). Inter-individual differences of the enantiomeric ratio (+/−) of tramadol were observed. Two of five volunteers excreted racemate during the period of investigation (Table III, volunteers C and D). On the other hand, three subjects exhibited a time-dependent increase of the excretion of (+)-tramadol (Table III, volunteers A, B and E). All volunteers eliminated significantly more (+)- than (−)-N-demethyltramadol (Table IV). Four of five volunteers showed also a time-dependent increase of the elimination of (+)-O-demethyltramadol. The fifth subject, who excreted very small amounts of the phenolic metabolite **2**, eliminated predominantly the (−)-enantiomer of the O-demethylated metabolite **2** (Table V, volunteer E).

CONCLUSION

The described assays were sensitive, rapid, reproducible and suitable for the quantification and the determination of the enantiomeric ratios of tramadol (**1**), O-demethyltramadol (**2**) and N-demethyltramadol (**3**) in human urine samples.

ACKNOWLEDGEMENTS

The authors thank Dr. K. Šindelař (Prague, Czechoslovakia), supported by a stipend of the Deutscher Akademischer Austauschdienst (DAAD), for valuable support in the synthesis of the metabolites **2–4**, Dr. E. Frankus (Grünenthal, Stolberg, Germany) for racemic tramadol, Dr. G. Scriba for helpful discussions, the volunteers B. K., U. S., M. S. and M. H. for their cooperation, and the Deutsche Forschungsgemeinschaft and the Fonds der Chemie for financial support.

REFERENCES

1 E. Frankus, E. Friderichs, S. M. Kim and G. Osterloh, *Arzneim.-Forsch.*, 28 (1978) 114.
2 W. Lintz, S. Erlacin, E. Frankus and H. Uragg, *Arzneim.-Forsch.*, 31 (1981) 1932.
3 B. Elsing and G. Blaschke, *Arch. Pharm.*, 324 (1991) 719.
4 K. Flick, E. Frankus and E. Friderichs, *Arzneim.-Forsch.*, 28 (1978) 107.
5 H. Derendorf and E. R. Garrett, *Pharmakokinetik*, Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1987, pp. 126–127.
6 B. Testa and A. H. Beckett, *Pharm. Acta Helv.*, 49 (1974) 21.
7 H. K. Kroemer and S. Botsch, *Pharm. Ztg. Wiss.*, 3 (1991) 109.
8 B. Schmid, J. Preisig and A. Küpfer, *Clin. Pharmacol. Ther.*, 38 (1985) 618.
9 P. Dayer, J. Desmeules, T. Leemann and R. Striberni, *Biochem. Biophys. Res. Commun.*, 152 (1988) 411.
10 B. Elsing, *Ph. D. Thesis*, University of Münster, Münster, 1992.
11 H. K. Kroemer, G. Mikus, T. Kronbach, U. A. Meyer and M. Eichelbaum, *Clin. Pharmacol. Ther.*, 45 (1989) 28.