Journal of Chromatography, 488 (1989) 417–425
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4577

DETERMINATION OF THE THALIDOMIDE ANALOGUES 2-(2,6-DIOXOPIPERIDINE-3-YL)PHTHALIMIDINE (EM 12), 2-(2,6-DIOXOPIPERIDINE-4-YL)PHTHALIMIDINE (EM 16) AND THEIR METABOLITES IN BIOLOGICAL SAMPLES

K. WINCKLER*

Freie Universitat Berlin, Institut für Biophysik, Thielallee 63, D-1000 Berlin 33 (F.R G.)

K.D. KLINKMÜLLER

Krankenhaus Moabit, Zentrallabor, Turmstrasse 21, D-1000 Berlin 21 (F.R.G.)

and

H.-J SCHMAHL

Freie Universitat Berlin, Institut für Toxikologie und Embryonalpharmakologie, Garystrasse 7, D-1000 Berlin 33 (F.R G)

(First received April 14th, 1988; revised manuscript received November 3rd, 1988)

SUMMARY

A rapid and sensitive method for quantitative analysis of the thalidomide analogues EM 12 and EM 16 and their metabolites has been developed. Following an optional extraction, samples were analysed by reversed-phase high-performance liquid chromatography with ion depression. The recovery of the extraction procedures was 65–80%. The method has been applied to pharmacokinetic studies in small laboratory animals and in vitro model experiments.

INTRODUCTION

Thalidomide (TD; Fig. 1a) is an outstanding example of a therapeutic agent that produces little or no toxic effect in the adult human. Its adverse effect on human embryogenesis is perhaps one of the most specific biological processes involving a chemical agent [1–4]. The mechanisms of teratogenic action of TD and the TD analogue EM 12 (Fig. 1a), and the metabolites responsible, are still unknown.

The renewed interest in the use of TD as an anti-inflammatory agent, as well as a potent drug for the treatment of various diseases of the skin, including the lepra reaction (erythema nodosum leprosum, ENL) [5], requires sufficient information about side-effects of TD. Although there are structural similarities

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.

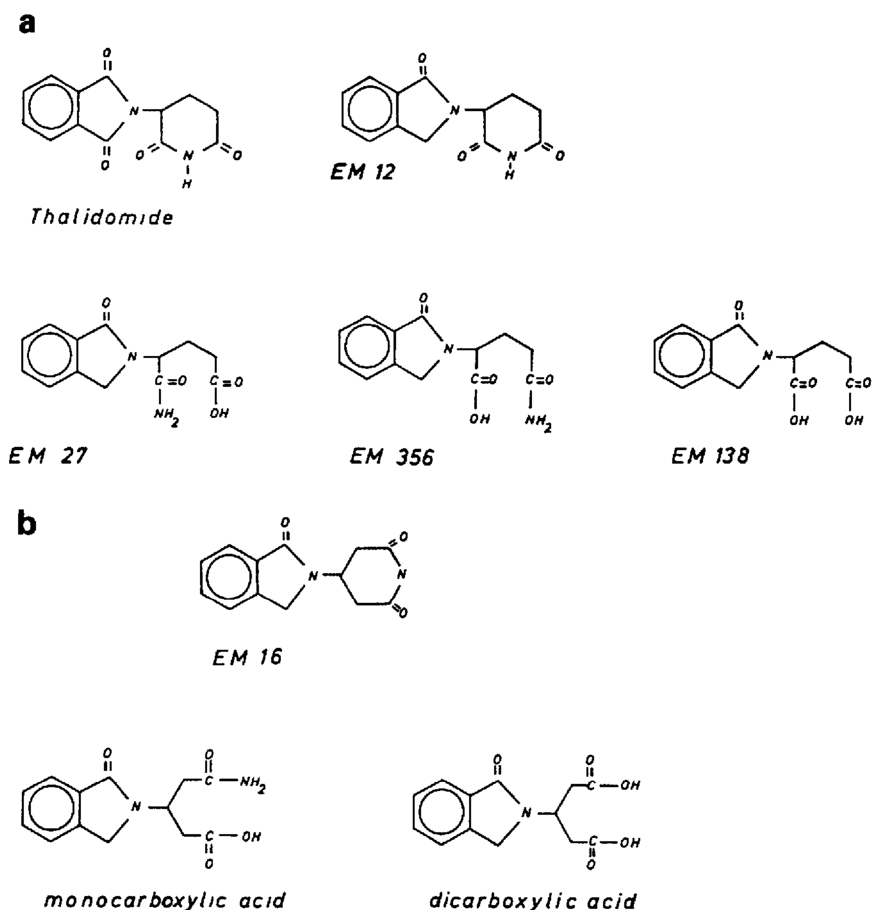

Fig. 1. Structures of (A) thalidomide and the teratogenic analogue EM 12 and its metabolites EM 356, EM 27 and EM 138, and (B) the non-teratogenic thalidomide analogue EM 16 and its metabolites.

between TD, EM 12 and EM 16 (Fig. 1), there are geat differences in their teratogenic potential and in the number of metabolites they produce (at least twelve from TD, three from EM 12; and just two from EM 16). This favours the use of EM 12 and EM 16 in investigations of the mechanisms involved in the teratogenic action of TD. Most of the metabolites of TD, EM 12 and EM 16 are carboxylic acids, formed by hydrolysis of the parent compounds (see Fig. 1a and b) in aqueous medium at pH > 7 [6–9]. EM 12 exhibits a smaller therapeutic effect, but a higher toxic and teratogenic potential than TD [10,11]. The pattern of malformations and the sensitive phase during pregnancy for the teratological action of EM 12 are reported to be equivalent to the corresponding effects of TD in the rabbit and in the marmoset monkey, which is a "TD-sensitive species". In the rat, a "TD-resistant species", EM 12 also causes malformations, although the type is different from that caused by TD in the rabbit [12]. For EM 16 no teratogenic effects could be detected in different species.

Several paper or thin-layer chromatographic methods [6–9], but only one high-

performance liquid chromatographic (HPLC) method, [13] have been described for the determination of TD and its metabolites in biological samples. As we needed a rapid, sensitive and more simple method for our investigations of the action of EM 12 and EM 16, we developed the method described in this paper.

EXPERIMENTAL

Materials

2-(2,6-Dioxopiperidine-3-yl)phthalimidine (EM 12) (non-labelled and ^{14}C labelled, specific activity: 90 MBq/mg; chemical purity: > 98%), 2-(2,6-dioxopiperidine-4-yl)phthalimidine (EM 16), N-phthalimidino-glutaric acid 1-amide (EM 27), 3-(2,3-dihydro-1,1-dioxido-3-oxo-1,2-benzisothiazol-2-yl)-2,6-dioxopiperidine (EM 8), N-phthalimidino-glutaric acid (EM 138) 2-(2,3-dihydro-1,1-dioxido-3-oxo-1,2-benzisothiazol-2-yl)-glutaric acid 1-amide (EM 270) and (N-phthalimidino-glutaric acid-5-amide (EM 356) were kindly obtained from Dr. E. Frankus (Chemie Grünenthal, Aachen, F.R.G.). Methanol (LiChrosolv) and other organic solvents (all from Merck, Darmstadt, F.R.G.) were used as received. β-Glucuronidase was from Boehringer-Mannheim (F.R.G.). Biological samples were drawn from Sprague-Dawley rats or Marmoset monkeys.

Sample preparation

Blood plasma. To 100 μl of plasma sample, 300 μl of hydrochloric acid–potassium chloride buffer (pH 1.6, 0.2 *M* hydrochloric acid and 0.2 *M* potassium chloride) including 10–20 μl internal standard [for EM 12, EM 8 in dimethyl sulphoxide (DMSO) (1 mg/ml); for EM 16, EM 270 in DMSO (1 mg/ml)] were added. The solution was saturated with solid sodium chloride and extracted three times with 500 μl of ethyl acetate. The extract was evaporated to dryness in a gentle stream of nitrogen and resuspended in DMSO.

Urine. Urine samples (100–500 μl, dependent on availability) were stabilized by adding twice the volume of a hydrochloric acid–potassium chloride buffer (pH 2.0; 21.2 ml of 0.2 *M* hydrochloric acid, 100 ml of 0.2 *M* potassium chloride, 278.8 ml water), including 10–20 μl of internal standard (as for plasma). The resulting solution was preprocessed using Sep-Pak C_{18} cartridges (Waters, Königstein, F.R.G.). Cartridges were preconditioned with 2 ml of methanol, followed by 5 ml of water. The sample was loaded to the cartridge and washed by adding 2 ml hydrochloric acid–potassium chloride pH 2.0 buffer, followed by 3 ml of buffer–methanol (8.5:1.5). The sample was eluted with 2 ml of methanol–water (7:3 for EM 16; 6:4 for EM 12) pH 7.0. An aliquot of the eluate was injected directly onto the columns.

Calibration curves for the determination of the substances from biological samples were set up by chromatographic analysis of certain concentrations of the single compounds.

Sample preparation for the detemination of EM 12, EM 16 and their metabolites from in vitro experiments was straightforward. If necessary, samples were deproteinized and stabilized by adding 4 volumes of ethanol–0.5 *M* phosphoric acid (4:1, v:v). Proteins were eliminated by centrifugation. The pH of the super-

natant was ca. 4.5. No further hydrolysis of relevant compounds could be detected up to 2 months when the samples were stored at $-20°C$. The supernatant was directly injected onto the analytical columns. Calibration curves for non-labelled compounds were set up by spiking the "stop-mix" with certain concentrations of the single compounds.

HPLC analysis

Two analytical columns (125×4.6 mm I.D., Knauer, F.R.G.) were coupled directly in the following order: 7 μm Nucleosyl-Phenyl (Macherey Nagel, F.R.G.) followed by 5 μm ODS-Hypersil (Shandon, U.K.). A Model 64.00 HPLC pump (Knauer), a Rheodyne 7125 injection valve fitted with a 20-μl loop, a Model SPD-2A spectrophotometric detector (Shimadzu, Japan) operated at 229 nm (for EM 12) or at 240 nm (for EM 16), a yt-recorder (JJ Instruments, U.K.) and an integrator (Shimadzu C-R3A) were used throughout the experiments. The eluent was methanol–50 m*M* phosphoric acid (21:79, v/v). The flow-rate was fixed at 1.1 ml/min. Analyses were carried out at room temperature.

Experiments using ^{14}C-labelled EM 12 were analysed by in-flow detection at 229 nm and scintillation counting after sampling. During a 30 min analytical run 59 samples were drawn using a fraction-collector (SuperRac, LKB, Sweden). Vial No. 60 contained an identical volume of the sample analysed for recovery studies. Vials were counted in a liquid scintillation counter (Model 1217 Rackbeta, LKB) for 10 min each after addition of 16 ml of scintillation cocktail (Unisolve 100, Zinsser, F.R.G.).

RESULTS AND DISCUSSION

Preparation of samples from biological fluids was necessary. Two sample preparation methods have been developed. Solid-phase extraction of urine samples with Sep-Pak C_{18} cartridges gave best results with respect to recovery and reproducibility for EM 12, EM 16 and metabolites (Tables I and II). This method is not recommended for the preparation of plasma samples, for which the extrac-

TABLE I

RECOVERY AND REPRODUCIBILITY OF EM 16 EXTRACTION FROM URINE SAMPLES

See text for details.

Compound	Recovery (mean ± S.D.) (%)	*n*
Monocarboxylic acid	82.5 ± 9.4	12
EM 16	77.3 ± 5.8	12
Dicarboxylic acid	Not detected	
Mean	79.9 ± 7 6	12
EM 270 (int. standard)	91.3 ± 8.7	3

TABLE II

RECOVERY AND REPRODUCIBILITY OF EM 12 EXTRACTION FROM URINE SAMPLES

See text for details.

Compound	Recovery (mean ± S.D.) (%)	n
EM 27	61.5 ± 4.1	4
EM 356	65.7 ± 4.3	4
EM 12	66.5 ± 2.7	4
EM 138	66.8 ± 5.9	4
Mean	66.1 ± 4.3	4
EM 8 (int. standard)	67.1 ± 3.9	4

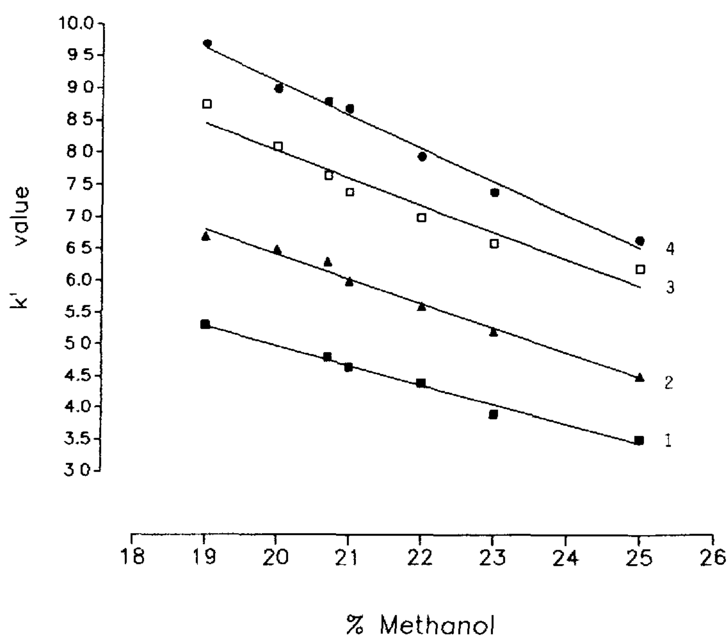

Fig. 2. Dependence of k' values of EM 12 and its metabolites on the concentration of organic modifier (methanol) in the HPLC eluent. Curves: 1 = EM 27; 2 = EM 356; 3 = EM 12; 4 = EM 138.

tion of EM 12, EM 16 and metabolites from a saturated sodium chloride solution by ethyl acetate gave recoveries similar to those for the solid phase extraction.

Samples from in vivo experiments with ^{14}C-labelled EM 12, as well as from enzymic reactions in in vitro experiments using EM 12 and EM 16, were deproteinized, and the pH was adjusted to less than 6, to prevent further alkaline hydrolysis of the compounds of interest. The use of deproteinizing reagents or mixtures other than ethanol–phosphoric acid can lead to unidentified reaction products ("artificial metabolites"). No further preparation of these samples was necessary.

Treatment of urine samples from rats and marmosets with β-glucuronidase (60

TABLE III

RETENTION TIMES AND CAPACITY FACTORS (k') OF EM 12, EM 16, THE MAIN METABOLITES AND THE INTERNAL STANDARDS

Given are the mean values at a methanol concentration in the eluent of 21% and a flow-rate of 1.1 ml/min at room temperature.

Compound	Retention time (min)	k'
EM 27	7.2	4.5
EM 356	9.1	6.0
EM 12	11.1	7.5
EM 138	12.8	8.8
EM 8 (int. standard)	14.8	10.3
Monocarboxylic acid	8.3	5.4
EM 16	12.7	8.8
Dicarboxylic acid	15.0	10.2
EM 270 (int. standard)	10.9	7.4

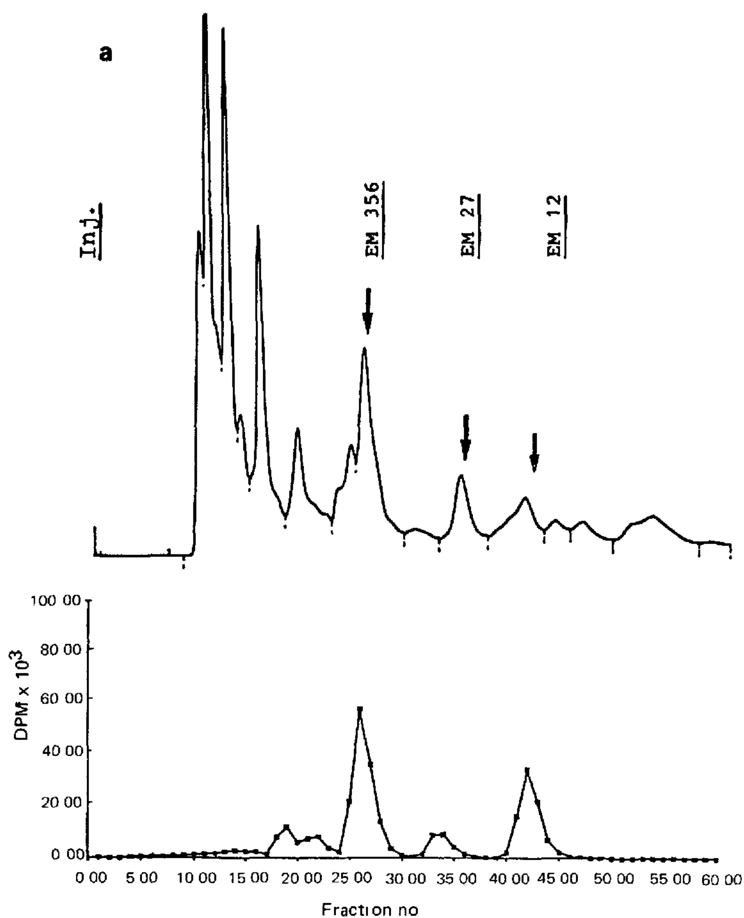

Fig. 3.

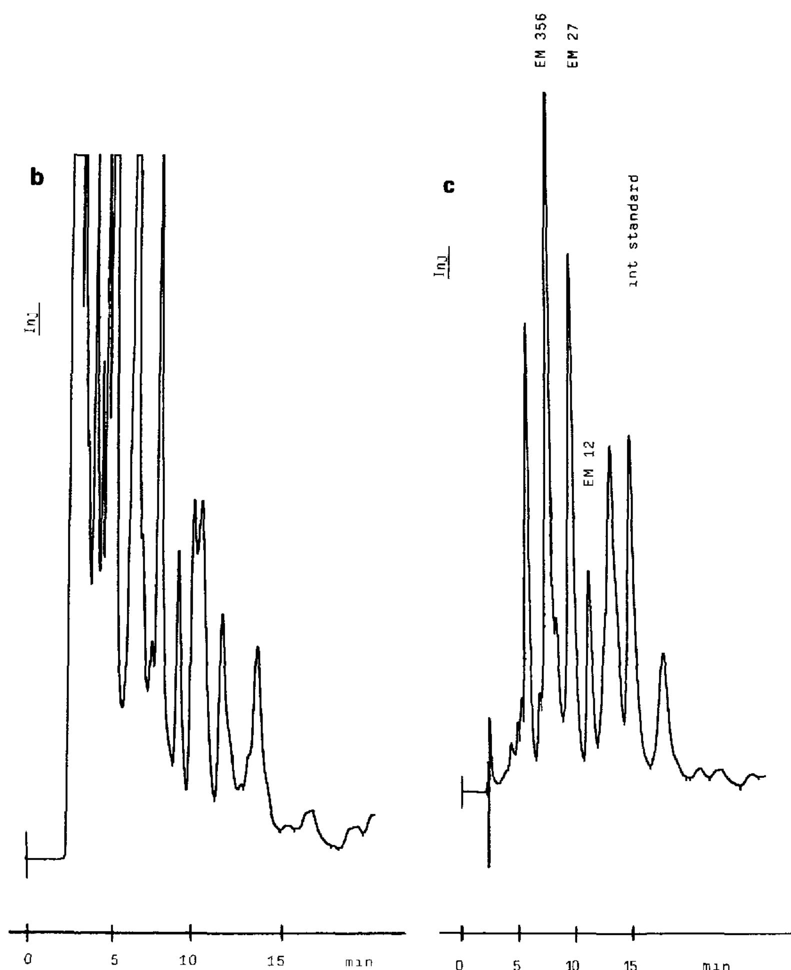

Fig. 3. Chromatograms of a urine sample drawn from a female marmoset 3 h after application of 40 mg of (A) ^{14}C-labelled or (B and C) unlabelled EM 12 per kg. (A) A 20-μl volume of the sample was injected directly onto the columns and analysed as described in Experimental, with UV detection at 229 nm. The lower part of the diagram shows detection by scintillation counting as described in Experimental. (B) A 10-μl volume of sample was injected without preparation directly onto the columns and analysed as described in Experimental, with UV detection at 229 nm. (C) A 200-μl volume of sample was prepared and 10 μl of the resulting extract were injected onto the columns and analysed as described in Experimental, with UV detection at 229 nm.

min at 37°C) before pH adjustment did not lead to any changes in the metabolic patterns or the relative amounts of the metabolites compared with samples in which the pH was adjusted directly. This indicates the absence of glucuronidized metabolic products of EM 12 in these species.

The analytical method presented offers a simple and fast tool for the qualitative and quantitative detection of EM 12 and EM 16 and their metabolites in a single run. Optimal results with respect to separation, analysis time and long-term stability of the stationary phases were obtained with the combination of the

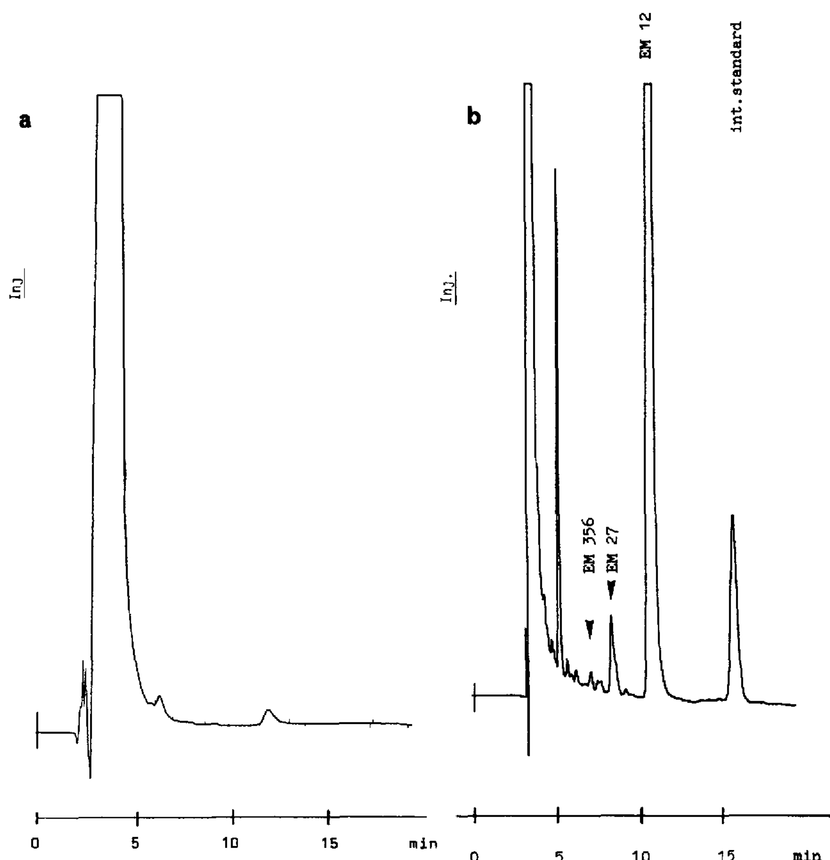

Fig. 4. (A) Chromatogram of 30 μl of blank plasma from a marmoset. The animal was not treated in any way, but the sample was prepared and analysed as described in Experimental, with UV detection at 240 nm. (B) Chromatogram of EM 12 and its metabolites from plasma of a marmoset. The sample (100 μl) was drawn 3 h after application of 20 mg of EM 12 per kg and prepared and analysed as described in Experimental, with UV detection at 240 nm.

two stationary phases described. The use of other stationary reversed phases gave no satisfactory results. The best eluent was methanol–50 m*M* phosphoric acid (21:79). The methanol content can be varied over a small range to shorten the analytical runs without significant loss of separation capability (Fig. 2). The relevant chromatographic data of the method are listed in Table III. Detection limits for individual compounds are in the range 1–2 μg/ml (signal-to-noise ratio 3:1). The method gives a linear response in the concentration range 2–100 μg/ml.

Maximum sensitivity is achieved at a detection wavelength of 229 nm. For rejection of undesired UV absorption in samples containing EM 16 and metabolites, the wavelength for UV detection can be set to 240 nm without significant loss of sensitivity.

With ^{14}C-labelled EM 12 we achieved a recovery rate of 98.8%. [injected, 11 225 $\pm$ 151.1 dpm; recovered, 11 091 $\pm$ 220.1 dpm ($n=6$)] for the HPLC method.

Figs. 3 and 4 show a set of typical chromatograms from experiments using EM 12.

The method has been applied to pharmacokinetic studies and the in vivo and in vitro metabolism of EM 12 [14] and EM 16 in female rats and marmosets.

ACKNOWLEDGEMENTS

We are indebted to Dr. E. Frankus and co-workers of Chemie Grünenthal GmbH for supplying us with materials for this study. The expert technical assistance of Ms. B. Guthmann is gratefully acknowledged. The work was supported by courtesy of Dr. H. Nau and by grants of the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 29).

REFERENCES

1 G.W. Mellin and M. Katzenstein, New Eng. J. Med., 267 (1962) 1184, 1238.
2 F.-Ch. Helm, E. Frankus, E. Friederichs, I. Graudums and L. Flohe, Arzneim. Forsch./Drug Res., 31 (1981) 941.
3 H. Koch, Sci. Pharm., 49 (1981) 67.
4 G.E. Shull, J. Theor. Biol., 110 (1984) 461.
5 H.P. Koch, in G.P. Ellis and G.B. West (Editors), Progress in Medicinal Chemistry, Vol. 22, Elsevier, Amsterdam, 1985, p. 165.
6 S. Fabro, H. Schumacher, R.L. Smith, R.B.L. Stagg and R.T. Williams, Brit. J. Pharamcol., 25 (1965) 352.
7 S. Fabro, R.L. Smith and R.T. Williams, Biochem. J., 104 (1967) 565.
8 S. Fabro, R.L. Smith and R.T. Williams, Biochem. J., 104 (1967) 570.
9 J.H. Knox and R.A. Hartwick, J. Chromatogr., 204 (1981) 3.
10 H.-J. Merker, W. Heger, K. Sames, H. Stürje and D. Neubert, Arch. Toxicol., 61 (1988) 165.
11 F.-Ch. Helm and E. Frankus, Teratology (Abstr.), 25 (1982) 47A.
12 A.J. Jackson and H.J. Schumacher, Canad. J. Pharmac. Sci., 15 (1980) 21.
13 M.J. Czejka and H.P. Koch, J. Chromatogr., 413 (1987) 181.
14 H.-J. Schmahl, K. Winckler, K. Klinkmüller, W. Heger, H.-J. Barrach and H. Nau, in H. Nau and W.J. Scott, Jr. (Editors), Pharmacokinetics in Teratogenesis, Vol. II, CRC Press, Boca Raton, FL, 1987 Ch. 12, p. 181.