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High-performance liquid chromatographic procedure for specificity testing of radioimmunoassays: rolipram

W. Krause* and G. Kühne

Research Laboratories of Schering AG, 1000 Berlin 65 (Germany)

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ABSTRACT

A high-performance liquid chromatographic (HPLC) procedure for testing the specificity of radioimmunoassays (RIA) was developed using the same method of extraction as in the RIA, followed by fractionation of the extract by HPLC and subsequent measurement of cross-reactions in all the fractions according to the normal RIA procedure. The RIA of rolipram, an antidepressant drug, was checked in plasma samples obtained from pharmacokinetic studies in rats, rabbits, cynomolgus monkeys and humans. The antibody was shown to be specific in the plasma samples from the laboratory animals, but not in human plasma. This was because in human plasma a metabolite occurred with a structure similar to that used for the hapten in the immunization process. This metabolite was not found in the plasma of the animal species investigated. The test procedure described is generally applicable, making the time-consuming development of an alternative method such as gas chromatography–mass spectrometry unnecessary.

INTRODUCTION

Radioimmunoassays (RIAs) are a very common technique used for the determination of many classes of compounds in biological fluids [1–3]. They are very sensitive and allow a high sample throughput. However, specificity is a general problem and is a source of error in many applications of RIAs [4]. Cross-reacting metabolites and/or endogenous compounds often significantly bias the results obtained by RIA. As a consequence, a second method is usually necessary to validate the data. The development of this second analytical technique, which is often gas chromatography–mass spectrometry (GC–MS) is time-consuming and tedious.

High-performance liquid chromatography (HPLC) is therefore proposed as a validating procedure for the separation of the analyte and the subsequent detection by RIA of cross-reacting compounds. This approach has been described previously for some compounds [5–7], but no systematic investigation has been performed.

Using rolipram (Fig. 1) as an example, the specificity of the RIA was studied in plasma samples from several species, including rats, rabbits, cynomolgus monkeys and humans.

Rolipram [4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone] is a selective cAMP phosphodiesterase inhibitor [8,9] which is effective in the treatment of endogenous depression [10,11]. The drug is well tolerated with no effects on the heart rate, blood pressure or other vital functions. Serotonergic or dopaminergic activities are not observed [12].

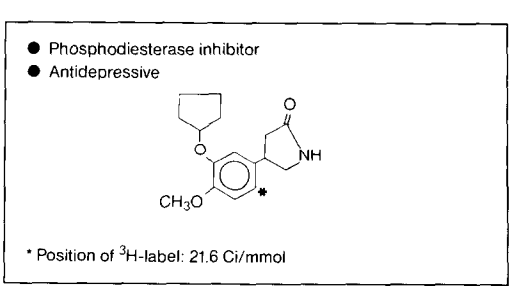

Fig. 1. Structure of rolipram. The position of the ^{3}H-label is marked by an asterisk.

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EXPERIMENTAL

Materials

Rolipram was labelled at position 6 of the phenyl ring with ^3H (Fig. 1). The specific activity was 2.9 GBq/mg (799 GBq/mmol). The radiochemical purity was greater than 99%. For use in animal and human studies the labelled drug was appropriately diluted with unlabelled rolipram.

Plasma samples

Plasma samples were obtained from rats, rabbits, cynomolgus monkeys and humans used in studies described previously [13,14]. The species, doses, time after administration of the drug and the number of specimens pooled to give the sample of interest are summarized in Table I.

Chromatographic procedure

The pooled plasma samples were divided into two portions. One portion (200–500 μl) was directly injected onto an HPLC column (LiChrosorb RP-18, 7 μm, 250 mm × 4.6 mm; Bischoff, Stuttgart, Germany) and separated using a linear gradient system from water with 0.1% acetic acid (v/v) to methanol with 0.1% acetic acid (v/v) in 30 min. The flow-rate was 1.5 ml/min. Two HPLC pumps (Type 510; Knauer, Berlin, Germany) and a gradient former (Type 680; Knauer) were used. The samples were injected with a Rheodyne RH 7120 injection valve (Waters, Königstein, Germany). Fractions were collected with an ISCO fraction collector (Lincoln, NB, USA) every 20 s. The radioactivity was determined in these fractions with a liquid scintillation counter (Type 610; Packard, Downers Grove, IL, USA).

TABLE I

CHARACTERISTICS OF PHARMACOKINETIC STUDIES FROM WHICH THE PLASMA SAMPLES WERE OBTAINED FOR RIA SPECIFICITY TESTING

Species	Dose (mg/kg)	Time post-administration (h)	*n*
Human	*ca.* 0.01	4	6
Rat	10	4	3
Rabbit	10	1	3
Cynomolgus monkey	10	2	3

The second portion of the samples was extracted with diethyl ether using the same procedure as applied in the RIA. An aliquot of 0.1–0.4 ml was extracted with 2.5 ml of diethyl ether after the addition of physiological saline to a final sample volume of 0.5 ml. The diethyl ether phase was

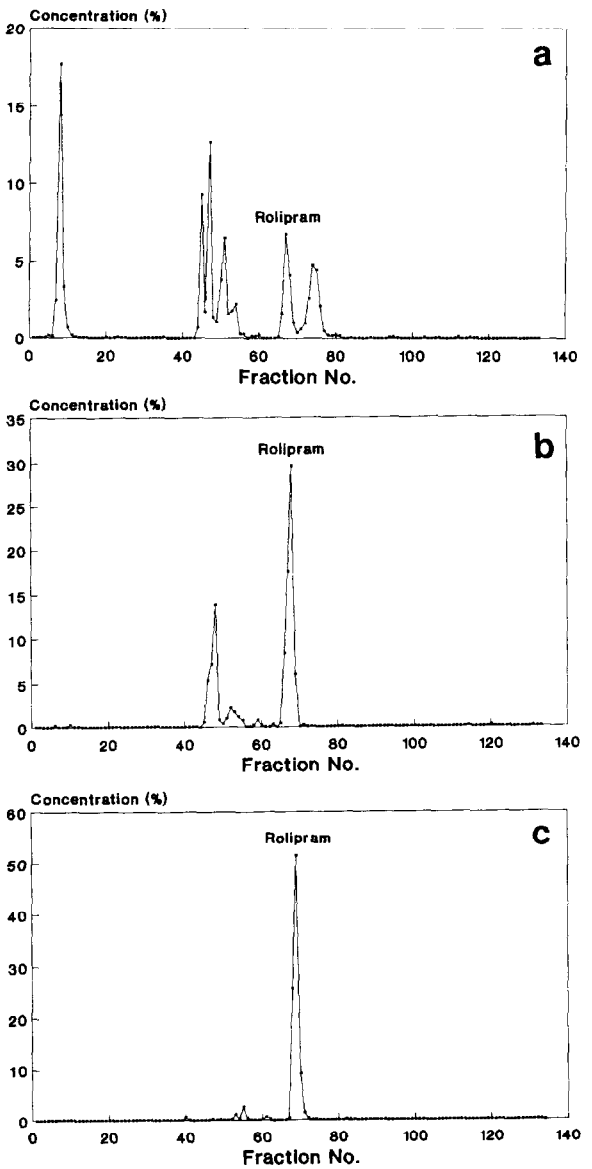

Fig. 2. High-performance liquid chromatograms of rat plasma obtained 4 h after oral administration of 10 mg/kg [^3H]rolipram. (a) Measurement of ^3H-activity in fractions from native plasma. (b) Measurement of ^3H-activity in fractions from plasma extracted with diethyl ether. (c) Measurement of RIA activity in fractions from plasma extracted with diethyl ether. Concentrations are given as percentage of total counts of the complete HPLC run.

evaporated to dryness and redissolved in 1 ml of aqueous bovine serum albumin solution (0.1%, w/v). This solution was then injected into the HPLC system and fractions were collected as described. The fractions were divided into two portions. The radioactivity was measured in the first portion and the RIA was performed on the second.

RIA procedure

The fractions obtained after HPLC separation were evaporated to dryness and redissolved in 1 ml of bovine serum albumin solution (0.1%, w/v). Incubation with a tracer and antiserum (1:250 000 dilution) was performed for 16 h at room temperature. Bound and unbound fractions were separated by a charcoal (Merck, Darmstadt, Germany) suspension containing 5% dextran T70 (Pharmacia LKB, Uppsala, Sweden). Following centrifugation (2100 *g*, 15 min), the radioactivity was measured in the solution. Calibration was achieved with a standard curve which was obtained parallel to the unknown samples. The antiserum was raised by immunization of rabbits with rolipram-3-carboxylic acid coupled to bovine serum albumin. The intra- and inter-assay variabilities were less than 3 and 7%, respectively. The detection limit of the assay was 0.1 ng/ml. The radioactivity of unchanged rolipram and/or of cross-reacting metabolites did not interfere with the measurement due to the low specific activity or was accounted for in the RIA evaluation programme.

RESULTS

Determination in plasma from rats

Approximately seven radiolabelled peaks could be separated by HPLC of native rat plasma (Fig. 2). One of the major peaks could be attributed to unchanged rolipram. A sulphate of rolipram hydroxylated in position 3 of the cyclopentyl ring (Fig. 3) could also be identified chromatographically [14]. Extraction with diethyl ether reduced the number of radiolabelled compounds to two plus three minor metabolites. The extraction efficiency was 33%. Rolipram was the dominant substance in the chromatogram. Detection by RIA in these fractions showed essentially one peak due to rolipram. The antibody is therefore assumed to be specific.

Determination in plasma from rabbits

Owing to the lower bioavailability of rolipram in the rabbit (about 4%), the peak in the native plasma chromatogram representing the un-

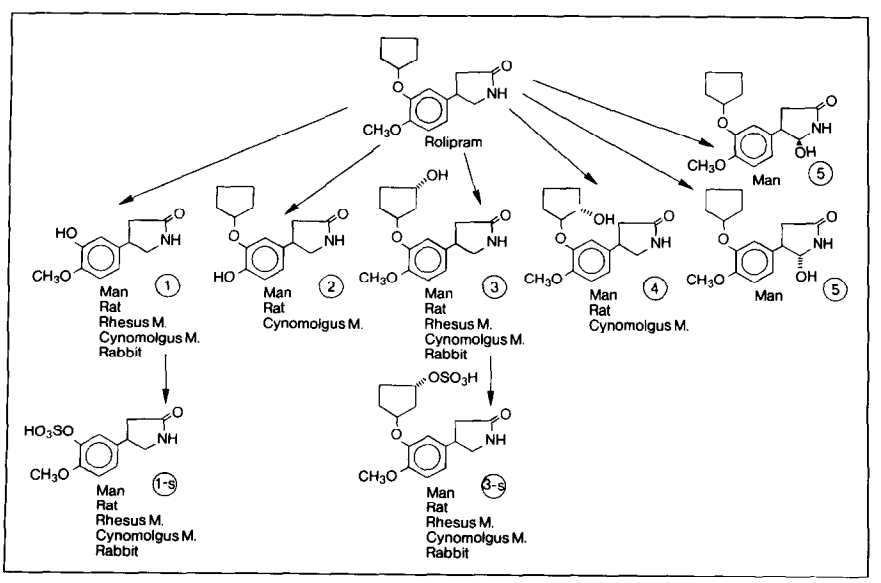

Fig. 3. Biotransformation pathway of rolipram in different animal species and in humans.

changed drug was only a minor peak. Approximately five metabolites were also present (Fig. 4). Extraction with diethyl ether with an efficiency of 5% eliminated the more polar compounds from the chromatogram, leaving rolipram as the dominant peak. Detection by RIA in these fractions showed only the unchanged drug and no metabolites. The RIA is therefore specific in rabbit plasma.

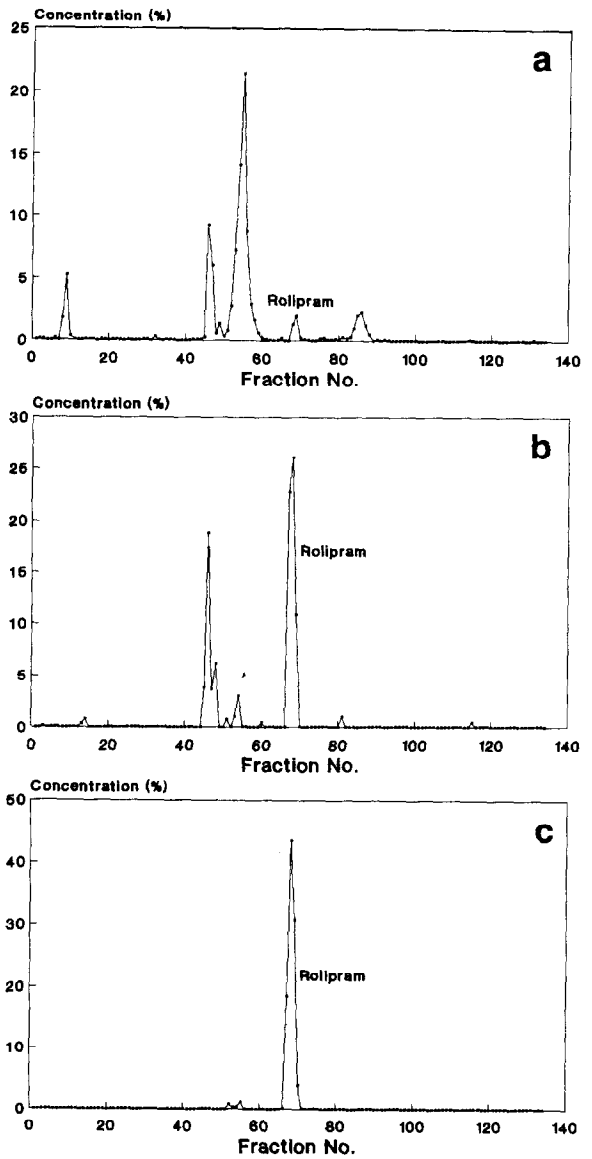

Fig. 4. High-performance liquid chromatograms of rabbit plasma obtained 1 h after oral administration of 10 mg/kg [^{3}H]rolipram. (a) Measurement of ^{3}H-activity in fractions from native plasma. (b) Measurement of ^{3}H-activity in fractions from plasma extracted with diethyl ether. (c) Measurement of RIA activity in fractions from plasma extracted with diethyl ether. Concentrations are given as percentage of total counts of the complete HPLC run.

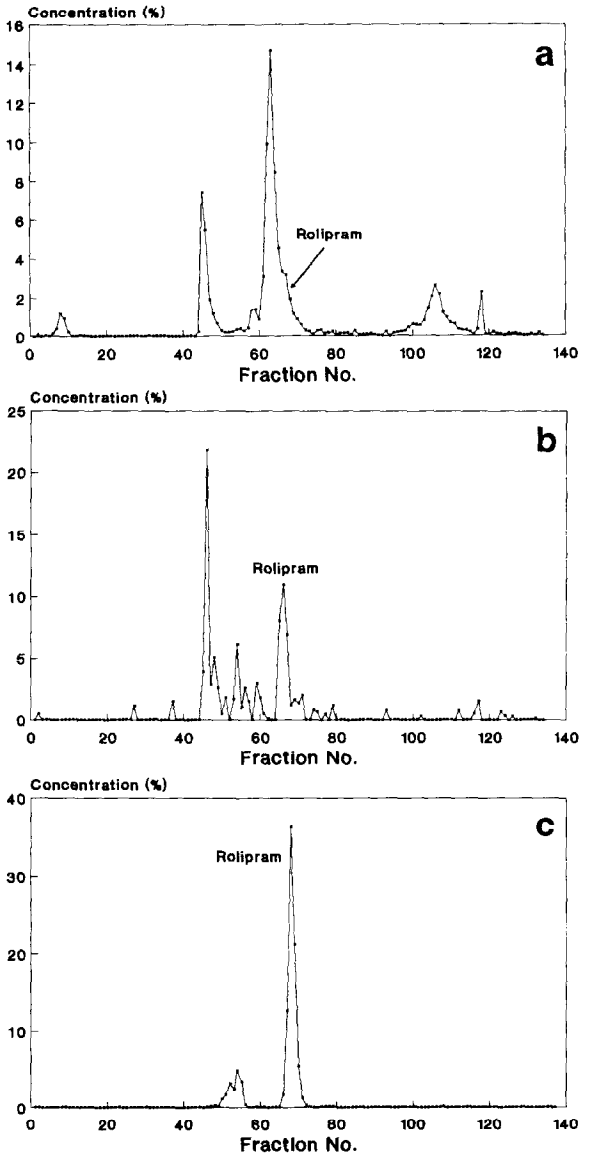

Fig. 5. High-performance liquid chromatograms of cynomolgus monkey plasma obtained 2 h after oral administration of 10 mg/kg [^{3}H]rolipram. (a) Measurement of ^{3}H-activity in fractions from native plasma. (b) Measurement of ^{3}H-activity in fractions from plasma extracted with diethyl ether. (c) Measurement of RIA activity in fractions from plasma extracted with diethyl ether. Concentrations are given as percentage of total counts of the complete HPLC run.

Determination in plasma from cynomolgus monkeys

In cynomolgus monkeys the bioavailability of rolipram was 4%, making it a minor compound seen with polar metabolites such as the sulphate of the hydroxy compound (Fig. 5). Extraction with diethyl ether with an efficiency of 10% eliminated this metabolite from the chromatogram. RIA detection showed a slight cross-reaction of two metabolites. However, the absolute value was negligible compared with rolipram.

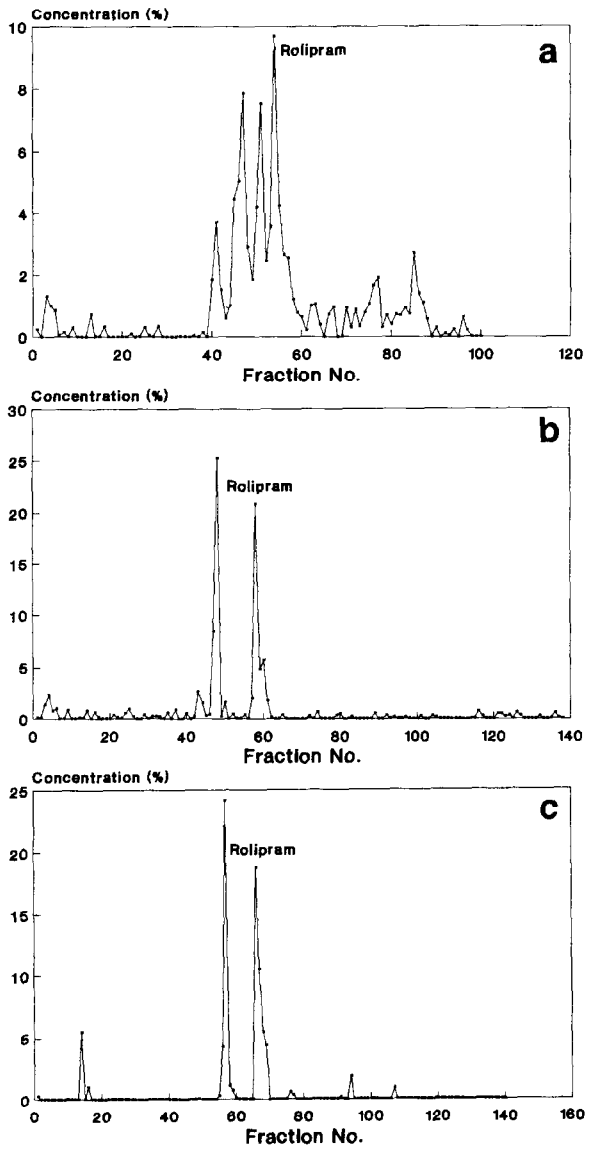

Fig. 6. High-performance liquid chromatograms of human plasma obtained 4 h after oral administration of about 0.01 mg/kg [^{3}H]rolipram. (a) Measurement of ^{3}H-activity in fractions from native plasma. (b) Measurement of ^{3}H-activity in fractions from plasma extracted with diethyl ether. (c) Measurement of RIA activity in fractions from plasma extracted with diethyl ether. Concentrations are given as percentage of total counts of the complete HPLC run.

Determination in plasma from humans

In human plasma a number of peaks were found in the HPLC profile obtained from native plasma (Fig. 6). Owing to the high bioavailability of 75%, the dominant peak was the unchanged drug. In addition, the sulphate of the hydroxylated metabolite of rolipram could be identified and also one metabolite, a derivative hydroxylated in the heterocyclic ring (Fig. 3), which has not been observed in any of the animal species investigated. Extraction with diethyl ether (recovery 60%) efficiently removed all polar compounds except for the heterocyclic hydroxyl metabolite. This compound also exhibited a high cross-reacting potential so that in the RIA detection chromatogram two peaks were observed: rolipram and the hydroxyl metabolite. The RIA, therefore, is not specific in human plasma.

DISCUSSION

In this study the specificity of the RIA for rolipram was tested using HPLC for the fractionation of the unchanged drug, cross-reacting metabolites and endogenous substances. The metabolite patterns of rolipram in various animal species and in humans have been extensively investigated and preliminary results have been reported previously [15]. Details of the isolation and identification procedure will be reported elsewhere [16]. One of the main metabolites observed in all the species investigated was a compound hydroxylated in the cyclopentyl ring of rolipram (Fig. 3) and its sulphate. In addition, demethylated substances and metabolites obtained by ether cleavage and subsequent conjugation were observed in all species investigated. However, one metabolite, a rolipram derivative hydroxylated in the heterocyclic ring, was found exclusively in human plasma.

For testing the specificity of the RIA, the usual RIA procedure was applied, which included extraction of the samples with diethyl ether. This

purification step effectively removed all the very polar metabolites such as sulphates of hydroxyl derivatives. The extraction efficiency of material obtained from the various species varied according to their content of conjugated metabolites. The lowest recoveries were found with rabbit plasma (5%) and the highest with human plasma (60%).

As a second "purification step" the samples were fractionated by HPLC and in all the fractions radioactivity and RIA activity were determined. This procedure showed that the antibody was specific towards rolipram in all the animal species investigated. However, in human plasma at least one cross-reacting compound was observed. This metabolite should be identical to the rolipram derivative resulting from hydroxylation of the heterocyclic ring system. This compound is exclusively formed in humans and is not seen in any of the animal species studied. At present it is unclear whether this metabolite is pharmacologically active. Cross-reaction of the rolipram antibody with this metabolite is feasible, because its structure is very similar to rolipram-3-carboxylic acid, which has been used for coupling to bovine serum albumin and immunization of rabbits to raise the antiserum.

Synthesis of the cross-reacting metabolite has not yet been successful. Therefore any information on the possible pharmacological activity of this compound is not available. If this metabolite is found to have an antidepressant activity, then the non-specificity of the RIA procedure will not be a problem in pharmacokinetic or drug monitoring studies because the data obtained would be representative of "active compounds". However, if this metabolite does not show any antidepressant effectiveness, there is a need for a new and specific method for determining rolipram in human plasma.

The procedure described for the testing of specificity seems to be generally applicable to all RIAs in use. It is easier to apply than the development of an alternative method of analysis such as GC–MS. HPLC is often not sensitive enough to compete with RIA as an assay and therefore is not suitable alone to check the specificity.

REFERENCES

1 W. Krause, R. Dorow, B. Nieuweboer and S.H. Hasan, *Eur. J. Clin. Pharmacol.*, 27 (1984) 335.
2 W. Krause, N. Sauerbrey and K. J. Gräf, *Eur. J. Clin. Pharmacol.*, 31 (1986) 165.
3 W. Krause and B. Nieuweboer, *Prostaglandins Leukotrienes Med.*, 14 (1984) 1.
4 E. Granström and H. Kindahl, in J.C. Frölich (Editor), *Methods in Prostaglandin Research*, Raven Press, New York, 1978, p. 119.
5 B. Nieuweboer, J. Tack, U. Täuber, M. Hümpel and H. Wendt, *Contraception*, 40 (1989) 313.
6 M. Hildebrand, B. Nieuweboer, H. Biere, U. Klar, G. Seemann, W. Krause and U. Jakobs, *Eicosanoids*, 3 (1990) 165.
7 W. Krause, G. Kühne and W. Seifert, *Drug Res.*, 41 (1991) 373.
8 H. Wachtel, *Psychopharmacology*, 77 (1982) 309.
9 H. Wachtel, *Neuropsychopharmacology*, 22 (1983) 267.
10 R. Horowski and M. Sastre, *Current Ther. Res.*, 38 (1985) 23.
11 E. Zeller, H. J. Stief, B. Pflug and M. Sastre-y-Hernandez, *Pharmacopsychiatry*, 17 (1984) 188.
12 E. Przegalinkski, K. Bigajska and A. Lewandowska, *Pharmacopsychiatry*, 14 (1981) 162.
13 W. Krause and G. Kühne, *Xenobiotica*, 18 (1988) 561.
14 W. Krause, G. Kühne and H. Matthes, *Xenobiotica*, 19 (1989) 683.
15 W. Krause and G. Kühne, *Xth International Congress of Pharmacology, Sydney, Aug. 23–28, 1987*, Abstract 0-88.
16 W. Krause and G. Kühne, in preparation.