Biochemical Pharmacology, Vol. 51, pp. 613–619, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02183-3

Determination of Rat Brain and Plasma Levels of the Orally Active $GABA_B$ Antagonist 3-Amino-propyl-n-butyl-phosphinic acid (CGP 36742) by a New GC/MS Method

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ABSTRACT. An involvement of GABAergic neurons has been suggested in the process of memory consolidation based on anatomical evidence and increasing physiological and biochemical data. With the advent of orally active $GABA_B$ antagonists, such as CGP 36742, the question of their therapeutic value, for example in Alzheimer's disease, becomes relevant. Therefore, a new GC/MS method was developed to determine the concentration of CGP 36742 (3-amino-propyl-n-butyl phosphinic acid) in various intra- and extracerebral tissues after different routes of application. The compound was chemically derivatised in a two-step process (acylation of the amino group and esterification of the phosphinic acid). The limit of detection of the method was 0.01 μg/g tissue and 0.0005 μg/mL plasma. The time-course after i.p. treatment showed peak levels of CGP 36742 between 30 min and 1 hr after injection. After a dose of 100 mg/kg, the concentration in the brain ranged from 1 to 1.4 μg/g or 6 to 8 μM, assuming that 1 mg tissue equals 1 μL (i.e., below the IC_{50} of the interaction with $GABA_B$ receptors as measured by [3-^{3}H]-aminopropyl-phosphinic acid binding [35 μM]). These results are discussed in light of the psychopharmacological effects (improvement of cognitive performance of rats) of CGP 36742 observed at very low oral doses. BIOCHEM PHARMACOL 51;5:613–619, 1996.

KEY WORDS. CGP 36742; $GABA_B$ receptors (blockade of); $GABA_B$ antagonists; gas chromatography/mass spectrometry; chemical derivatisation; rat brain; plasma

It was recently demonstrated that $GABA_B$ antagonists capable of crossing the rat blood-brain barrier selectively block central $GABA_B$ receptors [1, 2]. Neural $GABA_B$ receptors are located pre- and postsynaptically. Presynaptic $GABA_B$ receptors modulate the release of endogenous GABA and several other neurotransmitters. For instance, the $GABA_B$ antagonist CGP 35348 has been found to increase the release of GABA [3] and glutamate [4] in rat cerebral cortical slices. This compound also amplifies cholinergic signals in cortical neurons of rats [5]. Postsynaptic $GABA_B$ receptors mediate late IPSPs.† It has been shown that $GABA_B$ antagonists increase the responsiveness of target neurons by suppressing the IPSPs [6]. Thus, the main effect of $GABA_B$ antagonists consists of the amplification of neurotransmission. Therefore, it was hypothesized that they may enhance memory processing. Indeed, CGP 36742, the first orally active $GABA_B$ antagonist (IC_{50} = 35 μM determined by [3-^{3}H]aminopropylphosphinic acid binding [7]), improved the cognitive performance of mice (1–3 mg/kg; passive avoidance test), rats (3 mg/kg; partner-recognition test), and Rhesus monkeys (0.5 mg/kg; "conditional spatial color" task) [8].

In view of the relatively weak *in vitro* potency of CGP 36742, the doses exhibiting *in vivo* activity (in cognitive tests) seemed rather low. One may, then, expect a marked accumulation of the compound in the brain. However, the high hydrophilicity of CGP 36742 (log P n-octanol/phosphate buffer pH = 7.4, 20°C:–2.2) contradicts this hypothesis. To address this issue, the concentration of the $GABA_B$ antagonist in rat cortex, hippocampus, pituitary, adrenals, and plasma after i.p or p.o. administration was measured using a new GC/MS method. Moreover, we compared brain and plasma levels of rats treated acutely and subchronically to examine the possible accumulation of the drug.

The recently discovered $GABA_B$ antagonists contain the phosphinic acid moiety as a pharmacophore. Existing methods for the derivatization of amino acids are not applicable to phosphinic acids. Given the interesting pharmacological properties of this structural class of compounds, it is desirable to have a sensitive method for their detection, especially in the brain. Therefore, we developed a new derivatization process carried out in two steps: acylation of the amino group and esterification of the phosphinic acid.

Finally, the present study was aimed at evaluating any cor-

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† *Abbreviations*: C.V., coefficient of variation; IPSP, inhibitory postsynaptic potential; NCI, negative chemical ionisation; and SIM, selected ion monitoring.

Received 24 April 1995; accepted 4 October 1995.

relation between the concentration of CGP 36742 in the rat brain after administration of doses inducing an improvement of memory processing in different animal paradigms.

MATERIALS AND METHODS

Drugs and Chemicals

CGP 36742 (3-amino-propyl-n-butyl-phosphinic acid; MW = 179.2) and CGP 43792 (3-amino-propyl-n-propyl phosphinic acid; MW = 165.2) were prepared in the research laboratories of Ciba-Geigy Ltd. (Basel, Switzerland). Pentafluorobenzoyl-chloride and trimethylorthoformate were purchased from Fluka Chemie AG (Buchs, Switzerland).

Animals

Male Tif:RAIf(SPF) rats (Tierfarm Sisseln, Switzerland) weighing 160–200 g were used. They were housed in plastic cages in a room at a constant temperature of 21–22°C with a 12-hr light/dark cycle. The rats were fasted 24 hr before experiments.

Animal Treatment and Sample Preparation

Rats were sacrificed by decapitation at various intervals after treatment with CGP 36742. After dissection and weighing, brain areas (frontal cortices, hippocampi) were homogenised in 1 mL 0.1 M HCl containing 0.5 μg/mL of the internal standard CGP 43792. Pituitary and adrenals were homogenized in 0.5 mL of the internal standard solution. The homogenates were frozen at −80°C for 24 hr, and subsequently thawed and centrifuged for 15 min at 40,000 g. Plasma was prepared from trunk blood obtained at decapitation and frozen at −80°C. To 900 μL of thawed plasma, 100 μL of 2 M HCl containing 5 μg/mL of the internal standard were added (final concentration: 0.5 μg/mL).

Ten μL of 5 M NaOH and 10 μL of pentafluorobenzoyl-chloride were added to 100 μL of the supernatants or plasma and the mixture reacted at 22°C for 2 hr. After addition of 10 μL of 5 M HCl, the samples were dry-evaporated under vacuum at 50°C. Fifty microliters of trimethylorthoformate were added to each dried extract and the mixture reacted at 110°C for 2 hr. After evaporation to dryness under vacuum at 30°C, the residue was dissolved in 50 μL of MeOH and 0.5 μL was injected into the GC/MS.

Calibration Standards

A calibration curve was generated by adding graded amounts of CGP 36742 (0.0005–0.5 μg corresponding to final concentrations in standard homogenates of 0.0005–0.5 μg/mL) to 100 mg of brain tissue, homogenized in 1 ml 0.L M HCl containing 0.5 μg/mL of CGP 43792, or to the mixture of 900 μL of plasma and 100 μL of 2 M HCl containing 5 μg/mL of the internal standard. For each concentration, 5 aliquots were analyzed by the method described above. The ratio of peak areas of the derivatives of CGP 36742 and CGP 43792 were plotted against the concentration of CGP 36742 to calculate the parameters of the linear regression.

GC/MS Analysis

The GC/MS analyses were carried out on a Finnigan TSQ 700 mass spectrometer interfaced with a DEC station 5100 data processing system and coupled to a Fisons gas chromatograph model 8560.

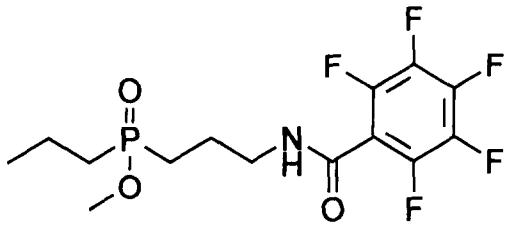
Gas chromatographic separation was achieved on a 12 m × 0.22 mm i.d. fused silica capillary column (SGE, 12 QC2/BPX5). The oven temperature was raised from 130°C at a rate of 10°C/min to 280°C. Helium was used as a carrier gas. The injection temperature was maintained at 290°C. The observed retention times were 9.9 min for CGP 43792 and 10.7 min for CGP 36742. The temperature of the GC/MS interface and the ion source were 290 and 150°C, respectively. The mass spectrometer was operated in the NCI mode with methane as the reagent gas with an ion source pressure of 8 torr. The filament current was 400 μA and the electron energy 70 eV. The conversion dynode and the multiplier were set at 15 kV and 1500 V, respectively. Quantitation was performed by SIM of the $[M_{derivative}\text{-}HF]^-$ ions for CGP 36742 (m/z 367) and CGP 43792 (m/z 353). The derivative structures are given in Fig. 1.

The limit of detection was between 0.01 and 0.02 μg/g tissue and approximately 0.0005 μg/mL plasma (defined as twice the background value).

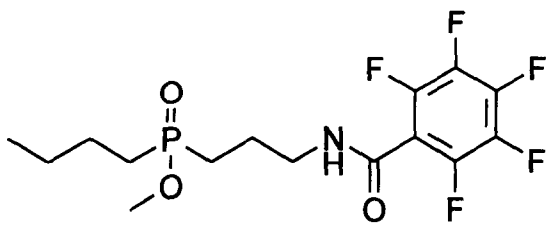
RESULTS

Mass Spectra and SIM Trace Chromatograms

The NCI (methane) mass spectra of the derivatives of CGP 43792 and CGP 36742 are shown in Fig. 2 (A and B). In both



Derivative of CGP 43792



Derivative of CGP 36742

FIG. 1. Structure of the derivatives of CGP 36742 and CGP 43792.

cases, the molecular ion $[M_{derivative}]^-$ lost HF (−20) to produce the $[M_{derivative}\text{-}HF]^-$ at m/z 353 and 367 for CGP 43792 and CGP 36742, respectively. These ions are the dominant peaks in the spectrum and were consequently chosen for SIM determinations. The $[M_{derivative}\text{-}2HF]^-$ are also present at m/z 333 and 347 for CGP 43792 and CGP 36742, respectively.

As shown by the SIM trace chromatograms in Fig. 3, no significant interfering peaks from the blank hippocampus extract (part A) were detected at the retention time of CGP 36742 (part B). Similar observations were made for CGP 36742 in plasma and for CGP 43792 in brain and plasma samples (results not shown). The concentration of CGP 36742 added to the hippocampus extract (part B) corresponds to a final level of 0.3 μg/g tissue (i.e., 30 times the limit of detection of the compound in the brain) (0.01 μg/g).

Precision and Linearity

Precision was characterized by the C.V., calculated for 5 aliquots of calibration samples containing graded concentrations

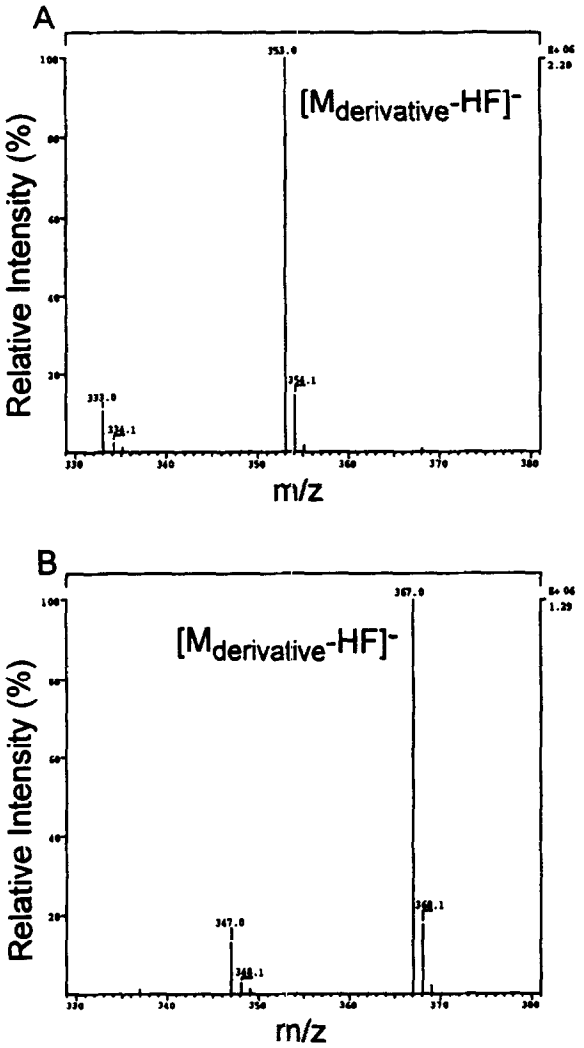


FIG. 2. NCI (methane) mass spectra of (A) the chemical derivative of CGP 36742 and (B) the chemical derivative of the internal standard CGP 43792.

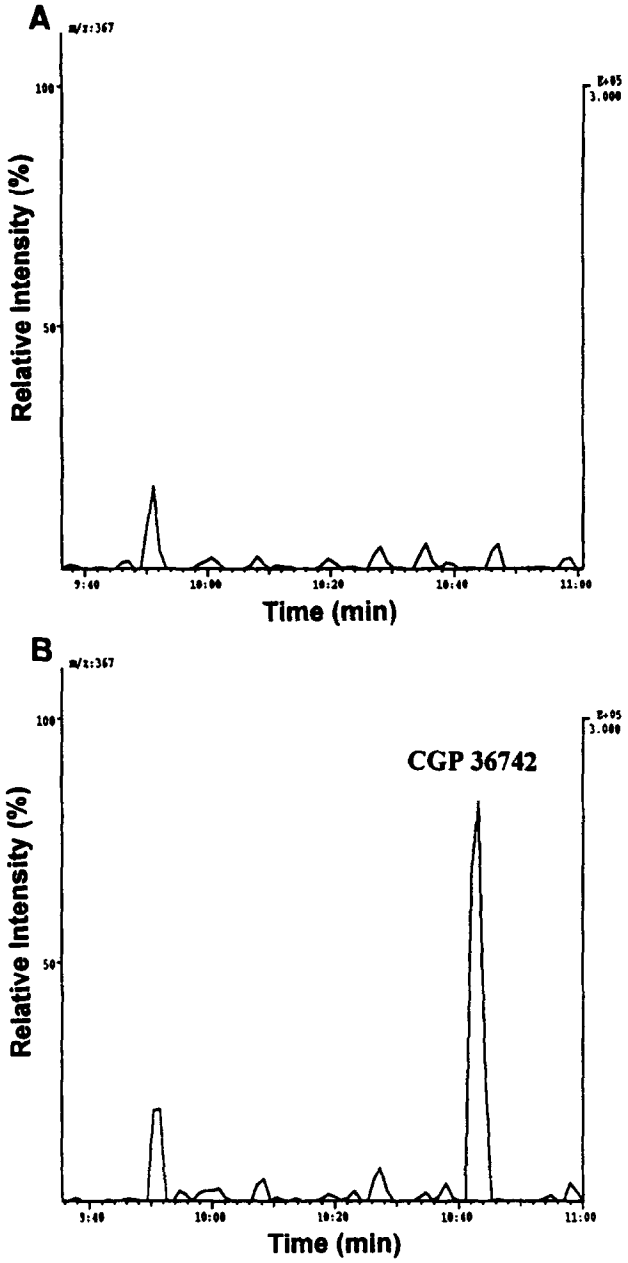


FIG. 3. SIM (m/z 367) trace chromatograms of (A) extract from 0.1 g of blank rat hippocampus and (B) extract from 0.1 g of rat hippocampus with 0.03 μg of CGP 43792 added. The samples were prepared and analysed as described in Materials and Methods.

of CGP 36742 (from 0.0005 to 0.5 μg/mL). It ranged from 4.6 to 11.3% (see legend of Fig. 4).

The mean calibration curve obtained for CGP 36742 was described by the following equation: $y = 5.49x + 0.0277$ (slope C.V.: 1%). The correlation coefficient (r^2) was 0.997 (Fig. 4).

Time-Course Experiment After Administration of 300 mg/kg i.p. of CGP 36742

The time-course after treatment with 300 mg/kg i.p. CGP 36742 showed peak levels between 30 min and 1 hr in the

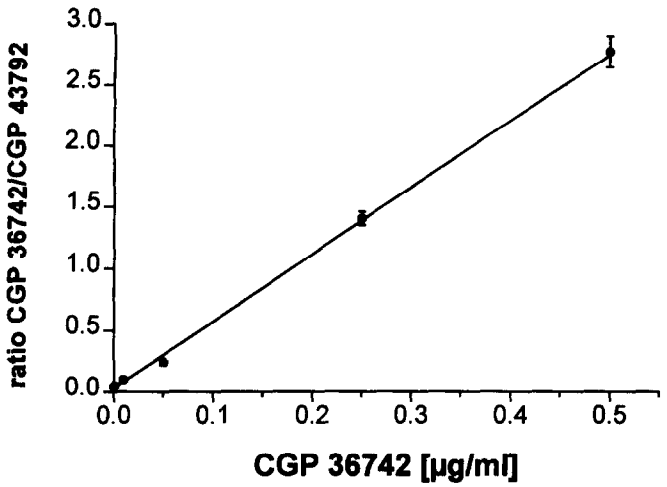


FIG. 4. Calibration curve of CGP 36742 in the brain. Graded amounts of CGP 36742 were added to 100 mg of brain tissue homogenized in 1 mL 0.1 HCl containing 0.5 µg/mL of CGP 43792. Five samples per concentration were analyzed as described in Materials and Methods. The ratio (mean ± SD) of peak areas of the derivatives of CGP 36742 and CGP 43792 were plotted against the concentrations of CGP 36742. The C.V. were calculated according to SD × 100/mean and were 11.3, 8.3, 9.9, 11.3, 6.9, and 4.6% for the solutions containing 0.0005, 0.001, 0.01, 0.05, 0.25, and 0.5 µg/mL of CGP 36742, respectively. Linear regression parameters: slope = 5.49 ± 0.06; Y-intercept = 0.0277 ± 0.0135; r^2 = 0.9968.

hippocampus (~1 µg/g) and in the adrenals (~24 µg/g) (Fig. 5). These levels fell rapidly (to ~0.3 and ~10 µg/g after 2 hr for hippocampus and adrenals, respectively) and then remained unchanged for up to 6 hr.

Time-Course Experiment After Administration of 300 mg/kg p.o. of CGP 3642

The time-course after treatment with 300 mg/kg p.o. did not show distinct peaks, particularly in the hippocampus, where the levels remained more or less constant from 1 to 5 hr after administration (approximately 0.3 µg/g) (Table 1). The highest concentration was measured after 2 hr in the plasma (16.6 µg/mL), pituitary (2.7 µg/g) and adrenals (2.6 µg/g) (Table 1). The concentration in a typical brain area, the hippocampus, was ten-fold lower than in the pituitary.

Time-Course Experiment After Acute or Subchronic Treatment with 100 mg/kg i.p. of CGP 36742

Rats were treated once or once daily for 10 days with 100 mg/kg i.p. of the compound to determine if there was any accumulation of the drug in the brain, pituitary, or adrenals. The experiment was carried out at day 10 of the subchronic treatment.

As shown in Fig. 6a, no significant difference was observed in the cortex between acute and subchronic treatment. Similarly, there was no significant difference between acute and chronic concentration profiles in the plasma (Fig. 7a): The tissue concentrations of the compound were slightly and significantly higher after subchronic treatment in the hippocampus at 2 and 24 hr (Fig. 6b), in adrenals at 4 hr (Fig. 7b), and in pituitary at 4 and 24 hr (Fig. 7c). In summary, there was no consistent accumulation of CGP 36742 after a 10-day subchronic treatment.

Levels of CGP 36742 After Administration of Graded i.p. Doses

The concentration of CGP 36742 was measured in the cortex, pituitary, and plasma 1 hr after administration of graded doses (0.1–100 mg/kg) (i.e., at the time at which peak levels had been observed in a previous experiment) (see Fig. 5). The results, given in Table 2, show that at 100 mg/kg a peak level of 1.0 ± 0.09 µg/g tissue was found in the frontal cortex. This maximum was 10 times higher in the pituitary (11.2 ± 1.0 µg/g tissue) and it reached 21.8 ± 2.0 µg/mL in the plasma. At 10 mg/kg, only 0.05 ± 0.02 µg/g tissue of CGP 36742 was detected in the cortex, and 0.69 ± 0.13 µg/g tissue and 0.96 ± 0.12 µg/mL were measured in pituitary and plasma, respectively. After administration of 1 mg/kg of the compound, 0.084 ±

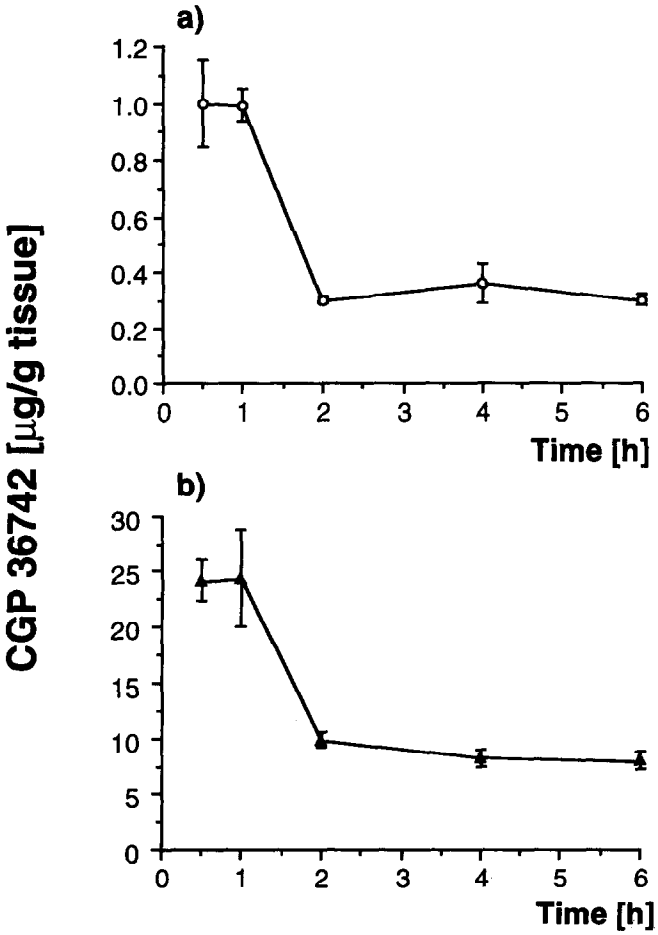


FIG. 5. Time-courses of the levels of CGP 36742 in hippocampus (a) and adrenals (b). Groups of 5 rats were treated with 300 mg/kg i.p. of the compound and decapitated at various intervals thereafter. The quantification was performed as described in Materials and Methods. Data are mean ± SEM in µg/g tissue.

TABLE 1. Time-course of the levels of CGP 36742 after oral administration of 300 mg/kg

Time after treatment (hr)	CGP 36742 Hippocampus (μg/g tissue)	Pituitary (μg/g tissue)	Adrenals (μg/g tissue)	Plasma (μg/mL)
1.0	0.24 ± 0.03	2.30 ± 0.36	2.10 ± 0.24	11.75 ± 1.03
1.5	0.30 ± 0.05	2.24 ± 0.34	2.04 ± 0.13	13.16 ± 1.39
2.0	0.26 ± 0.06	2.71 ± 0.34	2.60 ± 0.33	16.56 ± 1.34
3.0	0.24 ± 0.04	1.72 ± 0.23	1.25 ± 0.22	11.31 ± 1.23
5.0	0.22 ± 0.03	1.94 ± 0.28	1.30 ± 0.15	9.60 ± 1.07

Groups of 5 rats were treated with 300 mg/kg p.o. of CGP 36742 and decapitated at various intervals thereafter. The amounts of CGP 36742 were determined in the hippocampus, pituitary, adrenals, and plasma as described in Materials and Methods. Data are mean ± SEM in μg/g tissue or μg/mL plasma.

0.007 μg/mL were still detected in plasma. Finally, at 0.1–1 mg/kg, the levels of CGP 36742 in cortex and pituitary were below the detection limits of 0.02 and 0.01 μg/g tissue, respectively.

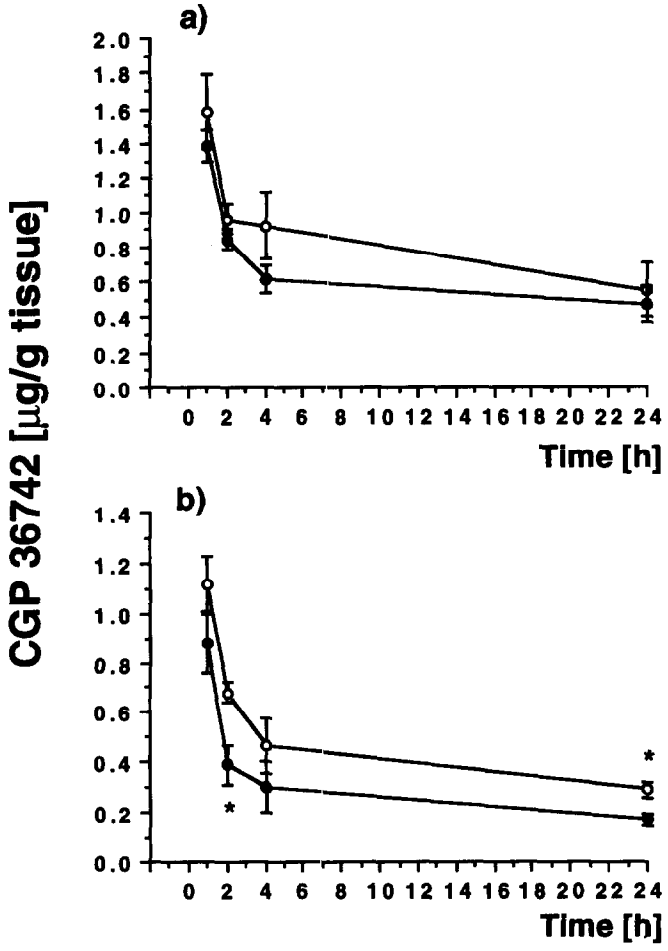


FIG. 6. Time-courses of the levels of CGP 36742 after acute (●) or subchronic (○) treatment in frontal cortex (a) and hippocampus (b). The rats (N = 5) were treated once or once daily for 10 days with 100 mg/kg i.p. of CGP 36742 and decapitated at various intervals thereafter. Data are mean ± SEM in μg/g tissue. Significance was assessed by comparing values of acute and chronic-treated animals at each time-point by Student's *t* test. *$P < 0.05$.

DISCUSSION

The GC/MS method developed to measure CGP 36742 in the brain and plasma was linear and reproducible. Esterification of the phosphinic acid was necessary to reduce the hydrophilicity of the tested compound and facilitate its elution through the GC column. Phosphinic acids do not lend themselves to the normal esterification methods employed for carboxylic acids. However, heating the pentafluorobenzyl amide of CGP 36742 (N-acyl derivative of the compound) with trimethylorthoformate produced the corresponding phosphinic acid methyl ester [9]. This method is easy to carry out and applicable to a large throughput of samples. It is sensitive enough to determine levels of the compound corresponding to 1% of the injected dose at 100 mg/kg, assuming homogeneous distribution of the drug.

The data demonstrate that CGP 36742 is rapidly available to the brain. After i.p. administration, peak levels were detected between 30 min and 1 hr postinjection. Similar amounts of the compound were found in the brain 1 to 5 hr after oral administration, indicating slow and constant absorption of the drug from the gastrointestinal tract. After oral administration, the maximal concentration of the drug present in the brain was approximately one third of that obtained after intraperitoneal injection.

No evidence for consistent accumulation of CGP 36742 in the brain, pituitary, and adrenals was found when the compound was administered daily at 100 mg/kg i.p. for 10 days.

The penetration of the rat blood-brain-barrier by CGP 36742 is low. The concentration of the compound measured in frontal cortex was only approximately 5% of the corresponding plasma concentration after 10 and 100 mg/kg i.p..

Moreover, when the peak levels obtained in the brain are expressed in μM, assuming that 1 mg tissue equals 1 μL, the concentration of CGP 36742 is below the value of the IC_{50} measured against [3-^{3}H]aminopropylphosphinic acid [7], (e.g., 0.3 μM CGP 36742 at 10 mg/kg i.p. and 6 to 8 μM at 100 mg/kg i.p. in the cortex). Assuming dose-brain concentration linearity, which appears justified from the brain levels obtained with 10 and 100 mg/kg i.p., brain levels are calculated to be 0.03 μM at a dose of 1 mg/kg i.p. Considering only the absolute mean concentrations of CGP 36742 after oral dosing of 10 and 100 mg/kg, the ratio of the respective brain levels

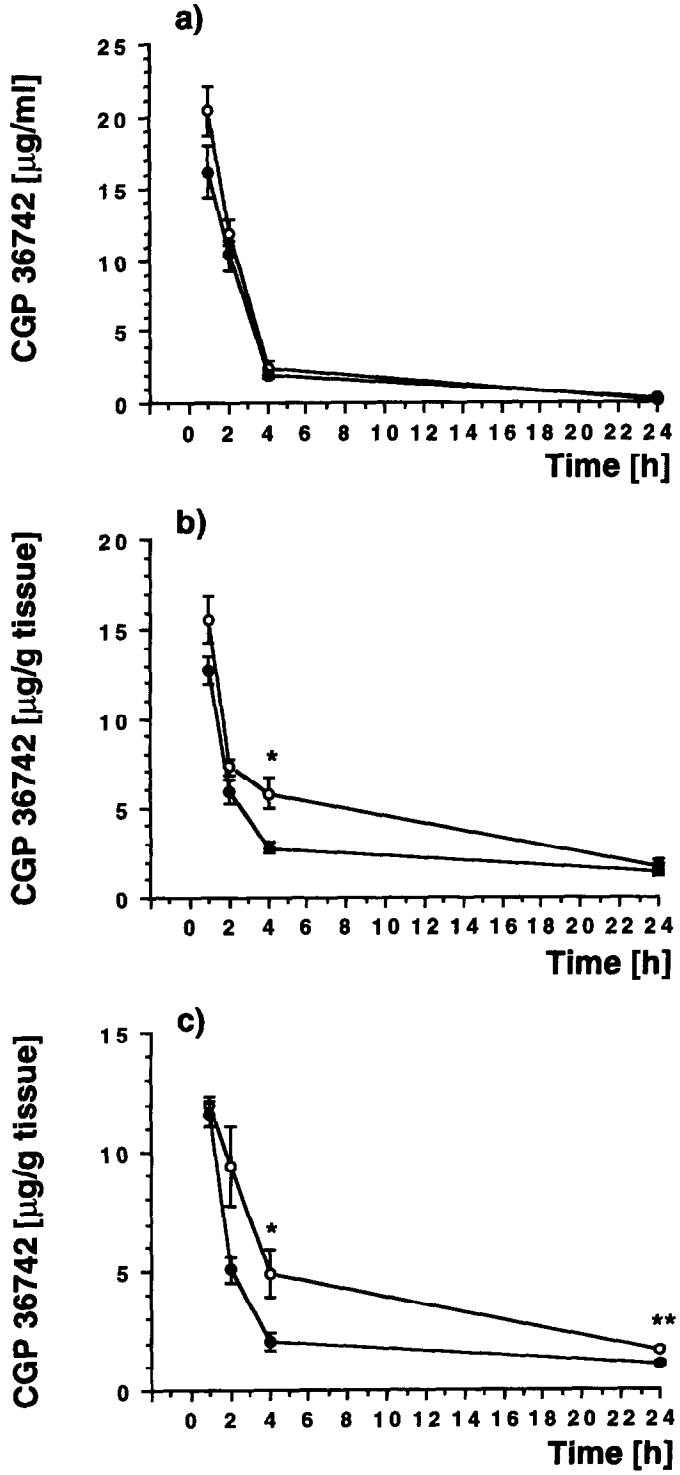


FIG. 7. Time-courses of the levels of CGP 36742 after acute (●) or subchronic (○) in plasma (a), adrenals (b) and pituitary (c). The rats (N = 5) were treated once or for 10 days once daily with 100 mg/kg i.p. of CGP 36742 and decapitated at various intervals thereafter. Data are mean ± SEM in µg/g tissue or µg/mL plasma. Significance was assessed by comparing values of acute and chronic-treated animals at each time-point by Student's *t* test. *$P < 0.05$, **$P < 0.01$.

TABLE 2. Levels of CGP 36742 after intraperitoneal administration of graded doses (0.1–100 mg/kg)

Dose of CGP (mg/Kg i.p.) 36742	CGP 36742		
	Frontal cortex (µg/g)	Pituitary (µg/g)	Plasma (µg/mL)
0.1	*	†	‡
0.3	*	†	‡
1.0	*	†	0.08 ± 0.01
10.0	0.05 ± 0.02	0.69 ± 0.13	0.96 ± 0.12
100.0	1.00 ± 0.09	11.17 ± 1.06	21.84 ± 2.03

Groups of 5 rats were treated with CGP 36742 and killed by decapitation 1 hr after injection. The concentrations of the compound were determined in the frontal cortex, pituitary and plasma. Data are mean ± SEM in µg/g tissue or µg/mL plasma.

* Level < 0.02 µg/g; †level < 0.01 µg/g; ‡level < 0.0003 µg/mL

appears to be 20. However, dose-brain level linearity can be postulated if the standard deviations of the absolute concentrations are taken into account, because this yields a ratio of 20 ± 10.

Positive effects on learning and memory (partner-recognition test) in the rat were observed at a dose of 3 mg/kg p.o. [8]. Therefore, our results suggest that the observed psychopharmacological effects are not a consequence of the blockade of brain $GABA_B$ receptors as defined by [3-^{3}H]aminopropylphosphinic acid binding, the IC_{50} for the inhibition by CGP 36742 being 35 µM in this assay.

However, CGP 36742 antagonizes the inhibitory effects of the $GABA_B$ agonist baclofen on spontaneously firing cortical neurons in a dose-dependent manner *in vivo*, the ID_{50} being 100 mg/kg p.o. [10]. Our results justify the assumption that a rat-brain concentration of 2 to 3 µM CGP 36742 is reached after administration of 100 mg/kg p.o. Thus, antagonism at central $GABA_B$ receptors is evident *in vivo*, despite the fact that the IC_{50} of 35 µM was not reached in the brain after this dose.

Therefore, there is an evident discrepancy between *in vitro* (concentration of CGP 36742 required for the blockade of $GABA_B$ receptors) and *in vivo* results (concentration measured in the tissue). One might suggest that CGP 36742 could be confined to the extracellular compartment in the brain, because it may penetrate cells only with difficulty. This would increase the estimated concentrations above in the vicinity of the $GABA_B$ receptors. It may be of interest in this context that the peak extracellular concentrations of CGP 36742 after intravenous administration of 100 mg/kg to rats in microdialysis experiments (R. Caprioli, personal communication; see also [11]) were similar to the peak tissue concentrations observed in the present study after 100 mg/kg i.p.. This argues against the compound being confined to the extracellular compartment of the brain.

Alternatively, it might be assumed that CGP 36742 interacts with other subtypes of $GABA_B$ receptors that are not adequately defined by the binding experiment with [3-^{3}H]aminopropylphosphinic acid. Indeed, there is increasing evidence for such a $GABA_B$ receptor family with pharmacologically distinct subtypes [4, 12, 13].

The authors wish to thank Anne-Marthe Buchle and Christine Grünenwald for their very helpful technical assistance.

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