Determination of (±)-1,2-Bis(3,5-dioxopiperazinyl)propane Plasma Levels in Rats, Rabbits, and Humans by GLC and Mass Fragmentography

W. SADÉE ˣ, J. STAROSCIK, C. FINN, and J. COHEN

Abstract □ Specific assay procedures were developed to measure plasma concentrations of (±)-1,2-bis(3,5-dioxopiperazinyl)propane (I) by GLC using flame-ionization detection with a sensitivity limit of 5 μg/ml and by GLC–mass fragmentography with a sensitivity limit of 0.2 μg/ml. Applicability of the assay procedures was demonstrated in rats, rabbits, and humans. Plasma concentration–time curves of total ^{14}C activity and intact I were obtained in rats and rabbits following oral and intravenous administration of ^{14}C-I. Plasma elimination half-lives of I in the first 2 hr following intravenous doses in rats were 40 and 45 min in two rats. Plasma levels of I were measured over 6 hr after an intravenous dose in rabbits and followed a two-compartment open model with a terminal log-linear plasma half-life of 85 min. Significantly higher total ^{14}C levels compared to intact I plasma concentrations indicated rapid biotransformation in both rats and rabbits to unknown metabolites. The oral bioavailability appeared to be limited in both species relative to intravenous administration. Two patients receiving 3 g of I/m^2 in tablets orally showed plasma levels of I similar to those obtained after oral doses in rats and rabbits, with peak concentrations at 2 hr after the dose (3.8 μg/ml) and with still measurable levels 12 hr after the dose (0.4 μg/ml).

Keyphrases □ (±)-1,2-Bis(3,5-dioxopiperazinyl)propane—determination of plasma levels, rats, rabbits, and humans, GLC and mass fragmentography □ Dioxopiperazines—determination of (±)-1,2-bis(3,5-dioxopiperazinyl)propane plasma level, rats, rabbits, and humans, GLC and mass fragmentography □ GLC—determination, plasma levels of (±)-1,2-bis(3,5-dioxopiperazinyl)-propane, rats, rabbits, and humans □ Mass fragmentography—determination, plasma levels of (±)-1,2-bis(3,5-dioxopiperazinyl)-propane, rats, rabbits, and humans

The dioxopiperazine, (±)-1,2-bis(3,5-dioxopiperazinyl)propane[1] (I, Scheme I) (1), is under clinical investigation as a chemotherapeutic agent against various neoplasms (2, 3). Its interesting pharmacological properties include normalization of tumor vasculature and the prevention of metastases of Lewis lung tumor in mice (4–6). The plasma disappearance half-life of I, following intravenous injections of ^{14}C-I in rats, was found to be about 30 min when measured by a bioassay using cultured Chinese hamster cells; total ^{14}C activity declined less rapidly, indicative of the presence of metabolites (7). The bioavailability of oral ^{14}C-I doses in humans appeared to be inversely related to the amount administered as judged by ^{14}C urinary excretion (8).

This article reports a specific chemical assay using GLC with flame-ionization detection and, for low concentrations of I, GLC–mass fragmentography. These methods were previously applied in other pharmacokinetic studies, *e.g.*, plasma levels of fluorouracil[2].

Rats and rabbits were utilized as experimental animals to demonstrate the applicability of the assay procedures, since pharmacokinetic data using the bioassay were available in rats (7) and evaluation of the effect of combination therapy with adriamycin and I on adriamycin cardiac toxicity is being investigated in rabbits. No intravenous preparation of I is yet available for human use due to limited solubility of the racemic mixture. The isolated optical isomers are considerably more soluble (9) and may provide an intravenous preparation in the future.

[1] Designated ICRF-159 by the Chemotherapy Department of the Imperial Cancer Research Fund.

[2] C. Finn and W. Sadée, to be published.

EXPERIMENTAL

Instrumentation and Apparatus—The assay was carried out on a gas chromatograph[3] equipped with a flame-ionization detector and a 1.8-m (6-ft) × 2-mm (i.d.) glass column packed with 3% polymethylsiloxane[4] on 100–120-mesh silanized diatomaceous earth[5]. Flow rates were approximately 35 ml/min for nitrogen, 30 ml/min for hydrogen, and 300 ml/min for air. The injector, column, and detector were operated at 210, 210, and 250°, respectively.

The GLC–mass fragmentography assay was performed on a gas chromatograph[6] equipped with an identical glass column and interfaced with a mass spectrometer[7] by a separator[8]. Helium, at a flow rate of 20 ml/min, was used as the carrier gas. The ionizing voltage was 70 ev.

Reagents—Chemicals were of analytical grade with the exceptions noted[9].

An ethereal alcoholic solution of diazomethane was prepared by the reaction of potassium hydroxide with *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[10] and stored at −10°. Because of its toxicity and explosiveness, all manipulations involving the generation or use of diazomethane were carried out in a well-ventilated hood behind a safety shield.

GLC—To 0.5 ml of plasma containing 5–50 μg of I were added 0.5 ml of saturated aqueous ammonium sulfate, 6.0 ml of ether–isopropyl alcohol (4:1 v/v), and 0.05 ml of a solution of 5 μg of methyl arachidate in methanol as an internal standard. Samples were mixed on a vortex-action high-speed mixer in stoppered test tubes for 4 min and centrifuged. The ethereal phase was pipetted into another test tube and evaporated to dryness with a stream of nitrogen at 40°. The residue was transferred to a 3.0-ml vial[11] with

[3] Varian Aerograph model 1400.

[4] OV-1.

[5] Gas Chrom Q.

[6] Varian Aerograph model 2700.

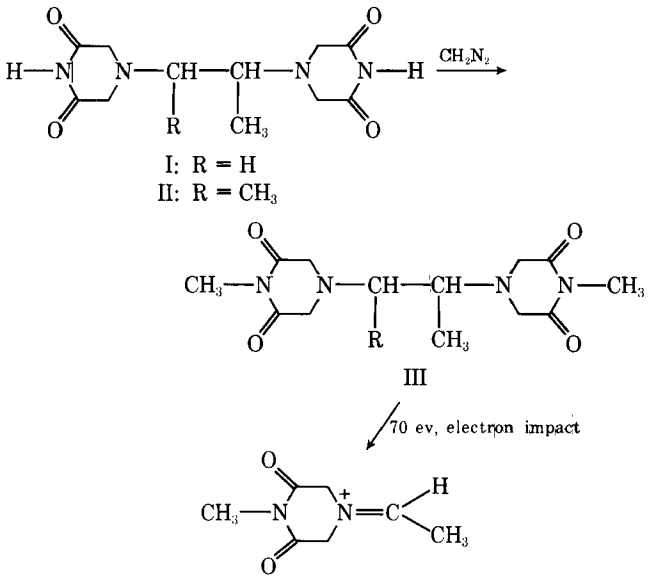
[7] Varian MAT CH-7.

[8] Biemann–Watson.

[9] Pure crystalline I (NSC-129943) was obtained from Dr. H. B. Wood, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Compound II was supplied by Dr. K. Hellmann, Imperial Cancer Research Fund, London, England. ^{14}C-Ethylene-labeled I was provided by Dr. M. A. Leaffer, Isotope Synthesis Lab, Menlo Park, Calif. (Contract NIH-NIC 71-2161).

[10] Diazald, Aldrich Chemical Co., Milwaukee, Wis.

[11] Reactivial, Pierce Chemical Co., Rockford, Ill.



Scheme I

3 × 0.5 ml of methanol, and the solution was again evaporated with a stream of nitrogen at 65°.

The residue was dissolved in 0.2 ml of methanol, and about 1.5 ml of diazomethane solution was added. The sealed vial was kept at room temperature for 4 hr, and then excess diazomethane was evaporated by allowing the opened vial to stand in the hood for several minutes. The remaining solution was evaporated to dryness with a stream of nitrogen at 65°. Care should be taken to thoroughly dry the residue at this point. The residue was dissolved in 0.1 ml of methylene chloride, and a 2–3-μl aliquot of this solution was injected into the chromatograph.

At a column temperature of 210°, methyl arachidate and the dimethylated derivative of I had retention times of 6.5 and 8.0 min, respectively. Both chromatographic peaks were sufficiently separated from each other and from interfering peaks in the methylated plasma extract to allow the determination of their relative heights. A plot of peak height of I/peak height of methyl arachidate *versus* concentration of I was linear over the 5–50-μg/ml range of plasma. The column must be reconditioned at about 260° for 15 min after each injection to elute less volatile plasma components.

GLC–Mass Fragmentography Assay—Extraction and derivatizing procedures were identical to those in the GLC assay using flame ionization. In place of methyl arachidate, 0.1 ml of a solution containing 1 μg of II in methanol was added as the internal standard[12]. Aliquots of 2–3 μl were injected on the column. The fragment ion at *m/e* 155 was used to monitor both methylated I and II (Scheme I). At a column temperature of 230°, the dimethylated derivatives of I and II had retention times of 4.0 and 5.0 min, respectively. A standard curve was again constructed from peak height ratios by assaying plasma samples containing known amounts of I in the 0.2–2.0-μg/ml range. A plot of peak height of I/peak height of II *versus* concentration of I was linear in this range.

Radioactivity Analysis—Plasma samples (0.1 ml) were counted for ^{14}C activity in 10 ml of scintillation fluid[13] in polyethylene vials. The counter[14] had a ^{14}C efficiency of 75–85%, and counts were corrected by the channels ratio method. The ^{14}C activity was expressed as equivalents of ^{14}C-I.

Animal Procedures—Female Sprague–Dawley rats, 250–300 g, were used. Blood samples were collected from a carotid cannula, spun in a microcentrifuge[15], and frozen for subsequent analysis.

[12] Compound II could not be used as the internal standard for the flame-ionization GLC assay because it could not be adequately resolved from interfering plasma components.

[13] Aquasol, New England Nuclear.

[14] Unilux II, Nuclear Chicago.

[15] Beckman.

Table I—Mass Spectra of *N,N'*-Dimethylated I and II Obtained by GLC–Mass Fragmentography

N,N'-Dimethylated I		*N,N'*-Dimethylated II	
m/e	Relative Abundance, %	*m/e*	Relative Abundance, %
58	22	58	22
70	37	70	33
99	22	99	21
127	52	127	44
141	7.6	155	100
155	100	156	16
156	28	—	—
296 (M^+)	4.4	—	—

Compound I was stable in frozen plasma samples over several weeks. New Zealand white rabbits, ~2 kg, were also used and blood specimens were obtained from an ear artery.

Doses of I were prepared in water, and sufficient 1.0 *N* HCl was added to dissolve all crystals completely. ^{14}C-I was added to obtain a specific activity of 100 dpm/μg. All doses were administered orally by stomach intubation or intravenously by injection over 1 min at a level of 120 mg/kg in rats and 50 mg/kg in rabbits.

Clinical Protocols—Human subjects received oral doses of I (3 g/m^2) in tablets as part of their medical treatment in the Western Cancer Study Group. Blood samples were obtained by venipuncture into containers[16] containing ethylenediaminetetraacetate and centrifuged, and the plasma was frozen for subsequent analysis. The use of this container resulted in an interfering peak with the same retention time as I, invalidating the flame-ionization GLC assay data. Plasticizers and similar compounds may have been possible contaminants. No interference by this contaminant was noted when utilizing single ion detection at *m/e* 155 in the GLC–mass fragmentography assay.

RESULTS

Assay Procedures—Chemical analysis of I imposed several analytical problems due to its polarity, amphoteric character, and lack of UV absorbance. The aqueous lipid extraction procedure, using saturated ammonium sulfate, yielded about 70% recovery of the drug from plasma samples. Suitable GLC characteristics were obtained by derivatization with diazomethane to give the *N,N'*-dimethylated derivative (III, Scheme I). The sensitivity of the flame-ionization GLC procedure was limited to 5 μg of I/ml of plasma due to interfering endogenous substances. Thus, single ion detection by mass spectrometry was employed to increase both specificity and sensitivity. Due to its close chemical similarity, Compound II was selected as the internal standard to compensate for extraction losses and irreversible adsorption processes during analysis at low concentrations.

The dimethylated derivatives of I and II are separated by GLC; both give rise to one major fragment ion at *m/e* 155 (Table I), which can be used to quantitate I and II. It is difficult to prove specificity of the assay for I in the presence of its metabolites since their structures remain unknown. The following criteria for assay interference by metabolites have to be met: extractability into the organic phase, identical GLC retention time, and fragment ion at *m/e* 155 representing a major peak in the mass spectrum. These criteria do not apply to possible hydrolytic polar metabolites. Therefore, the described assay is specific for I commensurate with current standards of plasma drug analysis.

The detection limit of pure samples of I by monitoring the fragment ion at *m/e* 155 was in the low picogram range. The detection of I in plasma samples after extraction was limited to approximately 0.2 μg/ml due to endogenous compounds with a high interference probability at the relatively low mass setting of *m/e* 155. However, this sensitivity limit was sufficient to measure I in rat, rabbit, and human plasma following therapeutic doses.

Plasma Levels in Rats—The plasma concentration–time

[16] Vacutainers.

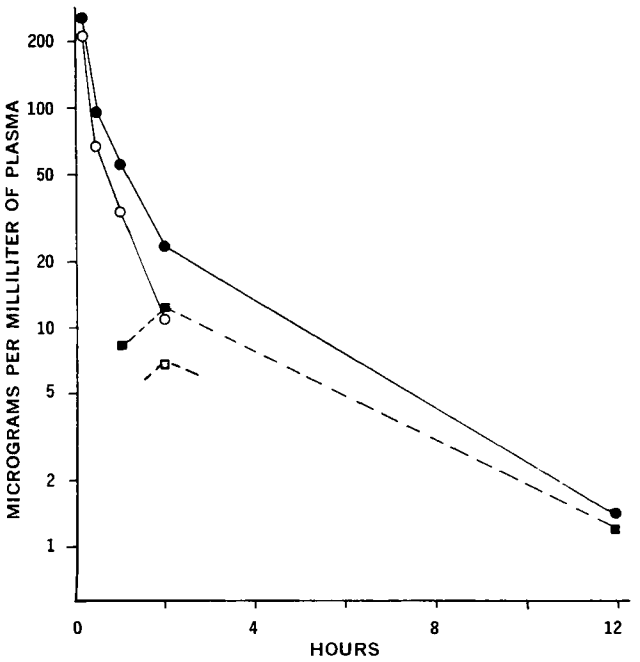


Figure 1—*^{14}C plasma activity and intact I plasma levels following a dose of 120 mg of I/kg in two rats. Key: ●, ^{14}C activity following intravenous dose; ■, ^{14}C activity following oral dose; ○, intact I levels following intravenous dose; and □, intact I levels following oral dose.*

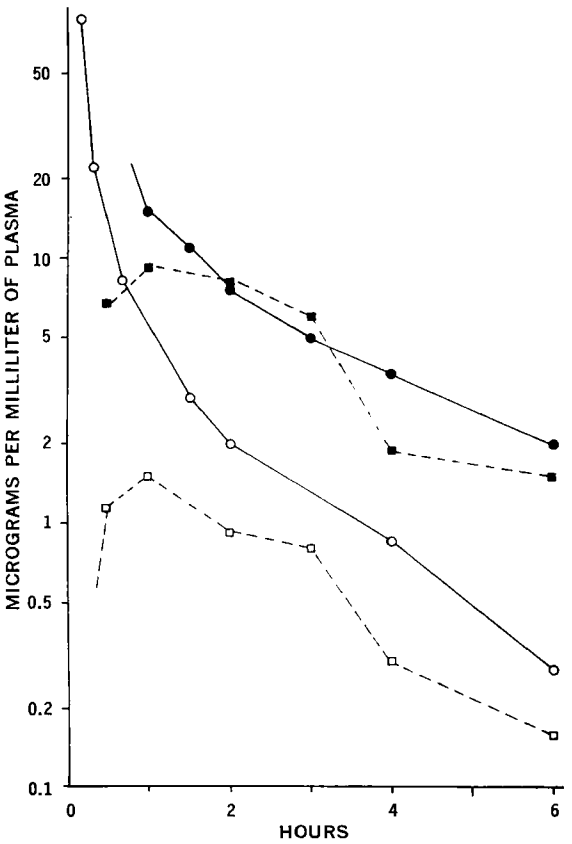


Figure 2—*^{14}C plasma activity and intact I plasma levels following a dose of 50 mg of I/kg in two rabbits. Key: ●, ^{14}C activity following intravenous dose; ■, ^{14}C activity following oral dose; ○, intact I levels following intravenous dose; and □, intact I levels following oral dose.*

curves measured by flame-ionization GLC and total ^{14}C activity resulting from an oral and intravenous dose of 120 mg of I/kg in two different rats are depicted in Fig. 1. Following intravenous administration, plasma levels of I rapidly disappeared during the initial distribution phase between 0 and 20 min and then declined with a half-life of about 40 min. A second rat showed a half-life of 45 min under identical conditions. The volume of distribution was obtained by extrapolating the apparent log-linear elimination phase to zero time and amounted to 120% of body weight in both rats. Total ^{14}C activity decreased less rapidly due to the formation of ^{14}C-labeled metabolites.

Following oral doses, peak plasma levels of 7 μg of I/ml were obtained after 2–4 hr, as measured by flame-ionization GLC (Fig. 1). Plasma levels of I were below the detection limit of this assay in all other samples.

Plasma Levels in Rabbits—^{14}C-I was orally and intravenously administered at a dosage of 50 mg/kg in two different rabbits (Fig. 2). Total ^{14}C plasma activity was again higher than the concentration of intact I, which was followed by GLC–mass fragmentation over 6 hr. The intravenous plasma concentration *versus* time curve of I could adequately be described by a two-compartment open model with a rapid α-phase ($\alpha = 4.15\ hr^{-1}$) and a slower β-phase ($\beta = 0.49\ hr^{-1}$, $T_{1/2} = 85$ min). The zero time intercepts were $A = 52$ μg/ml and $B = 5.5$ μg/ml.

Peak plasma concentrations of 1.5 μg/ml were obtained about 1 hr following oral administration and then declined parallel to the intravenous I concentration–time curve (Fig. 2), dropping below measurable levels 6 hr after administration. Total ^{14}C plasma activity remained as high as 0.6 and 0.5 μg/ml 24 hr after oral and intravenous administration, respectively, in these two rabbits.

Plasma Levels in Humans—The plasma concentration–time curve of I resulting from an oral dose of 3 g/m^2 (4.5 g) was measured by GLC–mass fragmentation (Fig. 3). Peak plasma levels of 3.8 μg of I/ml were obtained 2 hr following administration. Similar plasma concentrations were achieved in another patient under identical conditions. Twelve hours following administration of I, the plasma concentration in this patient had declined to 0.4 μg/ml and was below the detection limit (0.1 μg/ml) 24 hr after administration.

DISCUSSION

The specific assay for the determination of I in plasma samples was developed to evaluate the pharmacokinetics of this antineoplastic agent. The preliminary results are reported here.

Rat Studies—The plasma half-life of I measured by bioassay was about 30 min following an intravenous dose of 120 mg of I/kg (7). A plasma half-life of 40–45 min was found by the flame-ionization GLC assay under equivalent experimental conditions. Therefore, it is likely that no significant amounts of pharmacologically active metabolites were formed if one assumes that the bioassay is correlated with antineoplastic activity. Studies on the structure–activity relationships indeed demonstrate that pharmacological activity is very structure specific, and slight modifications of the chemical moiety of I usually abolish its antineoplastic activity (1, 10). However, it is difficult to assess pharmacological properties of I such as normalization of tumor vasculature, and formation of pharmacologically active metabolites cannot be ruled out.

The plasma half-life of 40–45 min for I may not represent the terminal log-linear elimination phase, since plasma levels were only followed over 2 hr by flame-ionization GLC.

Rabbit Studies—Plasma concentrations of I were measured by GLC–mass fragmentography over several half-lives. A log-linear β-phase for the elimination of I from plasma with a half-life of 85 min was reached 2 hr following intravenous administration. Initial plasma concentrations of I after intravenous injection were comparable to those obtained in rats when correcting for the lower dose in rabbits.

Plasma concentrations following oral doses were significantly lower than following intravenous doses in both species, indicating a limited oral bioavailability of I.

Human Studies—Only two patients were evaluated, each giving plasma concentration–time curves for I similar in order of magnitude to those obtained after oral doses in rats and rabbits. Therefore, it appears that both animal species may be useful as models for human pharmacological studies of I.

It cannot be determined at this point whether the limited bioavailability of I from oral preparations is due to low solubility or to

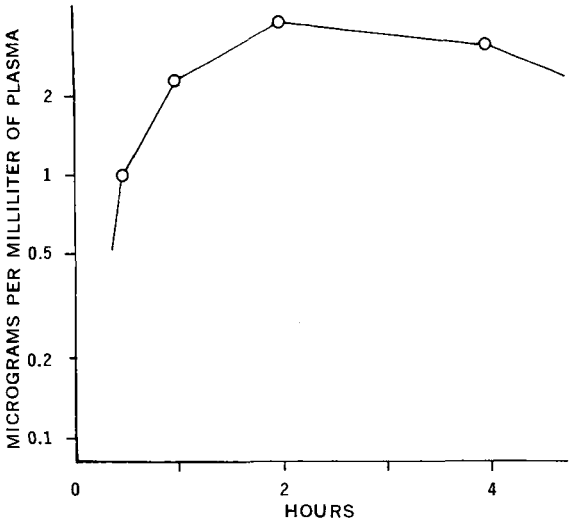


Figure 3—*Plasma concentrations of I in human subject following an oral dose of 3 g of I/m^2 in tablets.*

slow absorption through the GI mucosa. When given orally, I was slowly absorbed in all experiments, although an aqueous solution was used in rats and rabbits. The bioavailability of I is being studied.

REFERENCES

(1) A. M. Creighton, K. Hellmann, and S. Whitecross, *Nature*, **222**, 384(1969).

(2) R. E. Bellet, M. J. Mastrangelo, and J. W. Yarbro, *Cancer Chemother. Rep. (Part I)*, **57**, 185(1973).

(3) K. Hellmann, K. A. Newton, D. N. Whitmore, I. W. F. Hanham, and J. V. Bond, *Brit. Med. J.*, **1**, 822(1969).

(4) A. W. Le Serve and K. Hellmann, *ibid.*, **1**, 597(1972).

(5) A. J. Salsbury and K. Hellmann, *ibid.*, **4**, 344(1970).

(6) K. Hellmann and K. Burrage, *Nature*, **224**, 273(1969).

(7) E. D. Field, F. Mauro, and K. Hellmann, *Cancer Chemother. Rep. (Part I)*, **55**, 527(1971).

(8) P. J. Craeven, L. M. Allen, and D. A. Alford, "Abstracts of the 15th National Meeting of the APhA Academy of Pharmaceutical Sciences," San Diego, Calif., Nov. 1973.

(9) Minutes of the ICRF-159 Meeting, National Cancer Institute, Bethesda, Md., Nov. 8, 1973.

(10) A. M. Creighton and G. D. Birne, *Int. J. Cancer*, **5**, 47(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 8, 1974, from the *Clinical Pharmacology and Pharmacokinetic Sections and Western Cancer Study Group, School of Pharmacy and Medicine, University of Southern California, Los Angeles, CA 90007*

Accepted for publication December 2, 1974.

Supported by Public Health Service Grant CA05186-13 from the National Cancer Institute. Mass spectral analysis was in part supported by Public Health Service Research Grant CA14089-01 from the National Cancer Institute.

The authors gratefully acknowledge contributions to this study by Richard Brown and Howard Darvey.

[x] To whom inquiries should be directed. Present address: School of Pharmacy, University of California, San Francisco, CA 94143

Synthesis of 2-(*N*-Arylcarboxamide)-3-substituted Ethoxyindoles and Their Monoamine Oxidase Inhibitory and Anticonvulsant Activities

A. K. GUPTA [*], C. DWIVEDI [‡], T. K. GUPTA [*], SURENDRA S. PARMAR [*x], and RAYMOND D. HARBISON [‡]

Abstract □ 2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles were synthesized by the reaction of 2-(*N*-arylcarboxamide)-3-hydroxyindoles, which were obtained by the cyclization of 2-carbomethoxyphenylglycine-substituted anilides. These 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles were evaluated for their *in vitro* monoamine oxidase inhibitory ability and *in vivo* monoamine oxidase inhibitory property as evidenced by reserpine reversal response. Their anticonvulsant activity also was determined against pentylenetetrazol-induced seizures. No definite correlation could be observed between chemical structure and biological activity.

Keyphrases □ 2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles—synthesis, monoamine oxidase inhibitory and anticonvulsant activities □ Monoamine oxidase inhibitory activity—synthesis and screening of 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles, rat brain homogenate □ Anticonvulsant activity—synthesis and screening of 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles, mice

Identification of serotonin in the brain as a possible central neurohumoral agent (1) and the widespread occurrence of the indole nucleus among both naturally occurring and synthetic psychoactive compounds (2) stimulated an investigation of indole analogs with potent central nervous system (CNS) activity. In addition, clinical efficacy of 3-(2-aminobutyl)indole for the treatment of some types of depression (3) and the ability of indole derivatives to inhibit rat brain and rat liver monoamine oxidase (4, 5) and to protect against pentylenetetrazol-induced seizures (6, 7) prompted the synthesis of some 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles and their evaluation for CNS effects.