Journal of Chromatography, 144 (1977) 215–222

CHROM. 10,230

GAS–LIQUID CHROMATOGRAPHIC DETERMINATION OF CLONIDINE AND SOME ANALOGUES IN RAT BRAIN TISSUE

BRAIN CONCENTRATIONS AND HYPOTENSIVE ACTIVITY

P. B. M. W. M. TIMMERMANS, A. BRANDS and P. A. VAN ZWIETEN

Department of Pharmacy, Division of Pharmacotherapy, University of Amsterdam, Plantage Muidergracht 24, Amsterdam (The Netherlands)

(Received April 21st, 1977)

SUMMARY

A simple and sensitive gas–liquid chromatographic method has been developed for the quantitative determination of clonidine and some structurally related imidazolidines in rat brain tissue. The aqueous brain homogenates are first purified and then extracted into benzene. Samples are injected directly into the gas chromatograph. The extraction procedure is selective, and the use of a phosphorus–nitrogen detector enables accurate determinations corresponding to brain concentrations down to at least 10 ng/g. The rat brain concentrations of clonidine and its derivatives achieved at the moment of maximal decrease in arterial pressure are proportional to the doses administered intravenously, and are not influenced by the effect of the compounds on the blood pressure or by the method of anaesthesia employed. It is concluded that, for the linear part of the dose–response curves for these compounds, the brain concentration is a measure of the hypotensive effect.

INTRODUCTION

The antihypertensive drug clonidine (Catapresan®; 2-[2,6-dichlorophenylimino]imidazolidine hydrochloride) manifests its action via a central mechanism. This central hypotensive effect is presumably brought about by the excitation of central α-adrenergic receptors located at medullary sites[1–3]. The therapeutic action of clonidine is exerted after doses in the microgram range and, consequently, plasma concentrations and tissue contents are low. It has been possible to measure plasma and tissue levels after the administration of rather high doses of radiolabelled clonidine to humans and animals[4–7], by gas–liquid chromatography (GLC)[8–10] and by GLC–mass spectrometry (MS)[11]. These reports mainly aimed at a study of the fundamental pharmacokinetics of clonidine, and the GLC methods used were rather elaborate. Hitherto, the relation between the brain concentration and depressor effect of clonidine has been ignored, in spite of the fact that the therapeutic (hypotensive) action of this drug is of central nervous origin.

The present paper describes a convenient sensitive GLC method for the quantitative determination of clonidine and some of its structurally related imidazolidines in rat brain tissue. The compounds were administered intravenously, and brain concentrations were established at the moment of maximal decrease in blood pressure, in order to relate them directly to the hypotensive effect.

MATERIALS AND METHODS

Administration of the drugs; extraction from brain tissue

Male Wistar rats (weight 190–220 g) were anaesthetized with diethyl ether and surgically prepared in order to allow artificial respiration, injection of drugs via a jugular vein and measurement of arterial pressure via a carotid artery. Pressure was recorded via a Statham P23 Db transducer connected to a Hellige HE-19 recorder. During the operation, and throughout the experiment, the animal was kept under anaesthesia by continuous administration of diethyl ether ($6.1 \pm 0.2\%$, v/v, $n = 41$; for determination see *Gas–liquid chromatography*) to the inspiration air by means of a "Vapor" evaporator device (Drägerwerk, Lübeck, G.F.R.). After an equilibrium period of *ca.* 20 min, the blood pressure and heart rate had usually reached a constant level.

The imidazolidine of which the brain content was to be established was injected intravenously at a dose listed in Table I. At a preselected time after administration (see Table I), the animal was decapitated and the entire brain was removed immediately. The tissue was rinsed in saline to remove blood, gently blotted on filter paper and weighed. The brain was then homogenized in an aqueous solution of 0.02 *N* hydrochloric acid. The homogenate was transferred quantitatively to a 50-ml stoppered glass centrifuge tube and the volume was made up to 20 ml with 0.02 *N* HCl. Sodium chloride (1 g), benzene (10 ml) and ethyl acetate (10 ml, containing the internal standard) were then added. The tube was stoppered and shaken by hand for 5 min. After centrifugation for 10 min at 1000 *g*, the clear aqueous layer was isolated and again shaken with benzene (20 ml) for 5 min followed by centrifugation (10 min, 1000 *g*). 1 ml of an aqueous solution of 1 *N* sodium hydroxide was added to the isolated aqueous layer which was then extracted with benzene (20 ml). After centrifugation (10 min, 1000 *g*), the organic phase was pipetted off and evaporated to dryness under reduced pressure in a 10-ml conical glass tube. The residue was dissolved in 100 μl of benzene, and 1–10-μl samples were analysed by GLC.

Standards

Standard brain extracts were prepared by homogenation of the entire brains of untreated control rats in 0.02 *N* hydrochloric acid containing a known amount of the particular imidazolidine hydrochloride. The standards were then treated as described above.

Gas–liquid chromatography

Diethyl ether in the inspiration air was determined on a Packard Series 149 Becker gas chromatograph equipped with a flame ionization detector (temperature, 180°; hydrogen flow-rate, 30 ml/min; air flow-rate, 300 ml/min) and 1-mV Kipp

TABLE I

BRAIN CONCENTRATIONS (MEAN ± S.E.) OF TZ-1, TZ-13 AND TZ-18 AFTER INTRAVENOUS ADMINISTRATION TO ETHER-ANAESTHETIZED RATS

Assays of brain contents were performed at the moment of maximal decrease in blood pressure (t). The results for TZ-18 obtained under pentobarbital anaesthesia (see text) are marked with an asterisk (*).

Imidazolidine derivative	*Dose (μg/kg)*	*Number of determinations*	*t (min)*	*Brain concentration (ng/g)*
TZ-1	10	4	8	18.0 ± 2.6
(Clonidine)	20	4	10	39.7 ± 4.8
	35	4	15	64.2 ± 4.9
	50	3	20	91.5 ± 2.8
TZ-13	50	4	10	10.6 ± 2.0
(2-Cl,4-Me)	100	5	15	22.2 ± 2.1
	200	4	20	44.6 ± 4.2
TZ-18	20	3	4	60.6 ± 8.9
(2,4,6-tri-Cl)	20	3	4	57.1 ± 6.1*
	45	4	6	131 ± 7
	70	3	8	198 ± 12
	100	3	10	298 ± 8
	100	3	10	293 ± 10*

recorder. A glass column (2 m × 2 mm I.D.) packed with Porapak Q (80–100 mesh) was used at an oven temperature of 160° and an injector temperature of 180°. The carrier gas was nitrogen; flow-rate, 30 ml/min. Samples of inspiration air were injected directly. The gas chromatograms were evaluated by an integrating system from Spectra-Physics (Autolab system IV). The diethyl ether concentration (%, v/v) was determined by comparison with a standard solution in isooctane.

Extracts of rat-brain homogenates were analyzed on a Perkin-Elmer Series 3920 gas chromatograph equipped with a phosphorus–nitrogen detector (PND) and a 1-mV Kipp recorder. The setting and optimizing of the detector for this particular analytical problem was performed according to the procedure outlined by Kolb *et al.*[12] and by Hartigan *et al.*[13], which resulted in the following operation parameters: temperature, 280°; hydrogen flow-rate, 2 ml/min; air flow-rate, 100 ml/min. A glass column (2 m × 2 mm I.D.) packed with 3% OV-17 on Chromosorb 750 (80–100 mesh) was used at an oven temperature of 200–270° (depending on the derivative to be analyzed) and an injector temperature of 280°. The carrier gas was helium; flow-rate, 30 ml/min. Brain extracts obtained as described above were injected in benzene. The gas chromatograms were evaluated by an integrating system from Spectra-Physics (Autolab system IV).

Chemicals and drugs used

Chromosorb 750 (80–100 mesh) (Chrompack, Middelburg, The Netherlands); OV-17 (Packard, Delft, The Netherlands); Porapack Q (80–100 mesh) (Waters Assoc., Milford, Mass., U.S.A.); sodium chloride (Merck, Darmstadt, G.F.R.), benzene (Merck), diethyl ether (Brocacef, Maarssen, The Netherlands) and ethyl acetate (Merck) were distilled through a 100-cm Vigreux column before use. Clonidine

(Catapresan®; Boehringer, Ingelheim, G.F.R.), indicated by the code TZ-1; 2-(2,3-dichlorophenylimino)imidazolidine free base (TZ-9), 2-(2-chloro-4-methylphenylimino)imidazolidine hydrochloride (TZ-13), 2-(2,4,6-trichlorophenylimino)imidazolidine hydrochloride (TZ-18) and 2-(4-bromo-2,6-dichlorophenylimino)imidazolidine free base (TZ-21) were obtained by synthesis[14].

RESULTS

Standard curves were constructed by analyzing benzene samples to which the free bases of clonidine (TZ-1; 2,6-di-Cl), TZ-13 (2-Cl,4-Me) and TZ-18 (2,4,6-tri-Cl) had been added in different amounts. These three compounds were selected because they represent the major classes of phenyl-substituted imidazolidines. The standard curves passed through the origin and were linear up to at least 500 ng of the derivative injected. This finding indicated that there were no adsorption losses on the column and also demonstrated the linearity of the recording with the PND. Standard curves were also prepared from brain homogenates to which known amounts of the hydrochlorides of these three substances (25–400 ng) had been added. The internal standards employed were 2-(arylimino)imidazolidine bases selected on account of their retention times in order to ensure a convenient peak separation. TZ-9 (2,3-di-Cl) was used as internal standard for the determination of clonidine and TZ-13, and TZ-21 (2,6-di-Cl, 4-Br) was used as the internal standard for TZ-18.

The GLC peaks of these derivatives after extraction from brain homogenates were well-defined; no interfering peaks were observed when comparing the chromatograms with those obtained from extracts of blank homogenates. The reproducibility of the determinations was characterized by a relative standard deviation of *ca.* 3% when injecting the same sample repeatedly. The standard curve of clonidine is illustrated in Fig. 1. The relation between the relative peak area and the amount of clonidine added to rat brain homogenates is linear up to at least 400 ng. The same holds true for the curves of TZ-13 and TZ-18. This indicates that the extraction procedure is free from disturbances.

The concentration of the same three imidazolidine derivatives achieved in rat brain tissue after intravenous administration of different amounts was established. The procedure prior to isolation of the brain (see Materials and methods) was identical to that followed in order to quantify the hypotensive effect of these particular compounds[15]. These experiments were carried out on anaesthetized rats. However, due to the interference of the pentobarbital peak in the chromatograms, diethyl ether (*ca.* 6%, v/v; for determination see Materials and methods) was employed as the anaesthetic instead of pentobarbital. The doses chosen for the intravenous administration of the imidazolidines spanned the range of the dose–response characteristics corresponding to the hypotensive effect[15]. In order to relate the brain concentration directly to the hypotensive effect of the substances, the moment of maximal decrease in blood pressure was taken as the time at which these assays of brain content were performed. The particular preselected times were deduced from the response–time curves, established separately[15].

Clonidine (TZ-1; 10, 20, 35 and 50 μg/kg), TZ-13 (50, 100 and 200 μg/kg) and TZ-18 (20, 45, 70 and 100 μg/kg) were injected intravenously into rats and the brain contents of these derivatives, reached at the moment of maximal decrease in blood

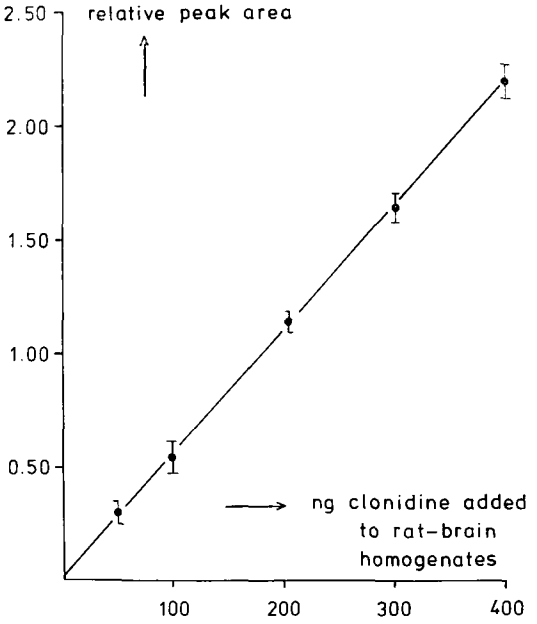

Fig. 1. Standard curve of clonidine. The compound was added in different amounts to rat brain homogenates with TZ-9 as an internal standard. Each point represents the mean value (± S.E.) from four experiments.

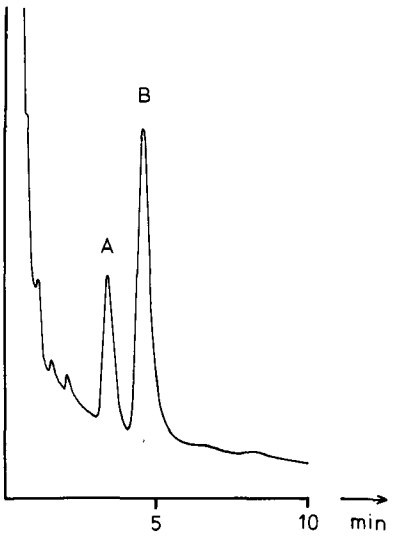

Fig. 2. Typical gas–liquid chromatogram obtained from an extract of rat brain homogenate. Peaks: A = clonidine; B = TZ-9 as an internal standard. The animal had received 10 μg/kg clonidine intravenously and the entire brain was removed 8 min after injection. The relative peak area of clonidine corresponded to 17.5 ng/g brain tissue (*cf.* Table I for the mean value from four experiments).

pressure, were established. The results are listed in Table I and are expressed as ng per g of brain tissue, calculated with the aid of the standard curves. Fig. 2 shows a typical gas–liquid chromatogram obtained from an extract of a brain homogenate of a rat which had received 10 μg/kg of clonidine (TZ-1) intravenously. This figure illustrates the selectivity of the extraction procedure and the sensitivity of the PND.

As can be seen from Table I, the method offers the possibility of the accurate determination of imidazolidines from extracts of brain homogenates corresponding to brain concentration levels down to at least *ca.* 10 ng/g. In principle, concentrations ten times lower than this should be easily detectable.

The brain concentrations of clonidine (TZ-1), TZ-13 and TZ-18, listed in Table I, are plotted in Fig. 3 against their corresponding intravenously administered doses. The curves pass through the origin and are linear. It may be concluded that the brain concentration reached at the moment of maximal decrease in blood pressure is proportional to the dose administered intravenously.

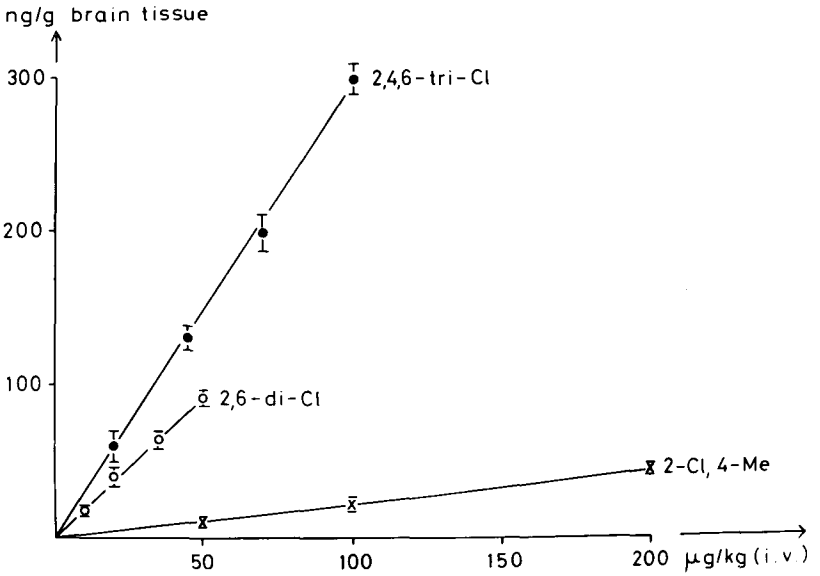

Fig. 3. Relation between brain concentration (ng/g, mean ± S.E.) of ether-anaesthetized rats and dose (μg/kg) administered intravenously for clonidine (2,6-di-Cl), TZ-13 (2-Cl,4-Me) and TZ-18 (2,4,6-tri-Cl). Brain contents were established at the moment of maximal decrease in arterial pressure. For numerical values see Table I.

The effects on the blood pressure of ether-anaesthetized rats after intravenous administration of clonidine and the two derivatives were considerably less than the responses measured when pentobarbital was used as the anaesthetic*. Thus the question arose as to whether the use of this volatile compound influences the disposition of the imidazolidines in the brain. This was investigated for TZ-18 which possesses a sufficiently long GLC retention time to enable an accurate determination of the

* It is outside the scope of this paper to go into a detailed discussion of these results, which are the subject of forthcoming investigations.

brain content without interference from pentobarbital in the chromatogram. In addition to the experiments described above, TZ-18 was given intravenously to rats anaesthetized with pentobarbital (75 mg/kg, intraperitoneal) at doses of 20 and 100 μg/kg. The brain concentrations of TZ-18 at the moment of maximal decrease in blood pressure are reported in Table I (marked with an asterisk), and are compared with the results obtained previously (ether anaesthesia) in Fig. 4. The results clearly demonstrate that the brain concentrations achieved by TZ-18 were virtually the same in ether and pentobarbital-anaesthetized rats. Furthermore, these data indicate that the results obtained with diethyl ether are directly comparable to those which would have been encountered if pentobarbital had been used.

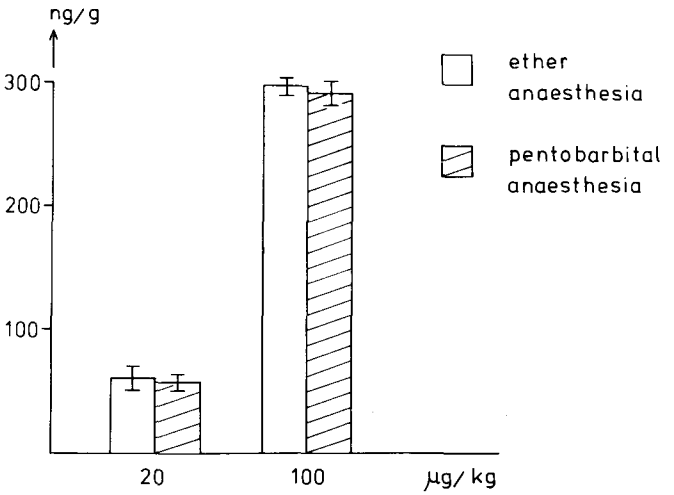

Fig. 4. Brain concentrations (ng/g, mean ± S.E.) of TZ-18 after intravenous application of 20 and 100 μg/kg to rats under pentobarbital (75 mg/kg, intraperitoneal) or ether anaesthesia (*ca.* 6%, v/v). Assays of brain contents were performed at the moment of maximal decrease in arterial pressure. For numerical values see Table I.

DISCUSSION

Apart from analytical procedures, which enabled radiolabelled clonidine to be determined directly from tissue and plasma[4–7], only a few GLC methods have been reported for the evaluation of clonidine. Cho and Curry[8] employed electron-capture GLC (GLC–ECD) after extraction. However, this method is not sufficiently sensitive to permit accurate measurements and tailed peaks are observed on different stationary phases. Concentrations of clonidine in plasma have been determined by selective ion monitoring after GLC–MS[11]. A GLC–ECD method has also been developed for the monotrifluoroacetyl derivative of clonidine[9]. However, in spite of the high sensitivity, this procedure is elaborate and impurities in the internal standard are also detected. Recently, Edlund and Paalzow[10] described a GLC–ECD procedure for clonidine in plasma, in which the drug was assayed as the pentafluorobenzyl derivative. This method is highly sensitive for clonidine, but is probably not generally applicable to the determination of related derivatives.

The procedure reported in the present paper is simple, the extraction from

brain homogenates is selective and the use of the PND enables accurate determinations of brain concentrations down to 10 ng/g.

The concentrations of TZ-1, TZ-13 (2-Cl, 4-Me) and TZ-18 (2,4,6-tri-Cl) in rat brain at the moment of maximal decrease in blood pressure were found to be proportional to the dose administered intravenously (Fig. 3). It therefore seems that the brain concentrations are not influenced by the effect of the compounds on the blood pressure. In addition, these results illustrate the importance of lipophilicity as a measure of the tendency of the three substances to penetrate into the brain. The lipophilicity, as determined by the partition coefficients ($\log P'$) between octanol and buffer (pH = 7.4), decreases from TZ-18 ($\log P' = 1.47$) via clonidine ($\log P' = 0.62$) to TZ-13 ($\log P' = -0.48$) and is paralleled by the decrease in the slopes of the lines in Fig. 3. Approximately 1.4% of the amount of clonidine administered intravenously is found in the brain, compared with *ca.* 2.3% for the more lipophilic TZ-18 and only 0.2% of the hydrophilic TZ-13. The use of diethyl ether as the anaesthetic instead of pentobarbital did not affect the brain content, as demonstrated for TZ-18.

The hypotensive effects of clonidine, TZ-13 and TZ-18 were quantified with the aid of dose–response curves following intravenous application to pentobarbital-anaesthetized rats[15]. The depressor activity of clonidine is very pronounced, whereas TZ-13 and TZ-18 are less potent. The dose–response characteristics have a sigmoid shape. For the linear part of the curves the logarithm of the dose administered intravenously is directly proportional to the depressor response. A linear relation also exists between the brain concentration and dose. Consequently, we submit that, for the linear part of the dose–response characteristics of clonidine and its derivatives, the logarithm of the brain concentration, assayed at the moment of maximal decrease in blood pressure, is directly related to the hypotensive effect.

REFERENCES

1 H. Schmitt, *Actual. Pharmacol.*, 24 (1971) 93.
2 W. Kobinger, in G. Onesti, K. E. Kim and J. H. Moyer (Editors), *Hypertension: Mechanisms and Management*, Grune and Stratton, New York, 1973, p. 369.
3 P. A. Van Zwieten, *Progr. Pharmacol.*, 1 (1975) 1.
4 D. Rehbinder and W. Deckers, *Arzneim.-Forsch. (Drug Res.)*, 19 (1969) 169.
5 D. Rehbinder, in A. Zanchetti and M. Enrico (Editors), *Ipertensione Arteriosa*, Boehringer, Ingelheim, 1973, p. 3.
6 J. P. Fillastre, D. Dubois and P. Brunelle, in A. Zanchetti and M. Enrico (Editors), *Ipertensione Arteriosa*, Boehringer, Ingelheim, 1973, p. 81.
7 S. Darda, in P. Milliez and M. Safar (Editors), *Recent Advances in Hypertension*, Boehringer, Ingelheim, 1975, p. 375.
8 A. K. Cho and S. H. Curry, *Biochem. Pharmacol.*, 18 (1966) 511.
9 A. Frydman, Y. Weiss, M. Safar and J. M. Alexandre, in P. Milliez and M. Safar (Editors), *Recent Advances in Hypertension*, Boehringer, Ingelheim, 1975, p. 369.
10 P. O. Edlund and L. K. Paalzow, *Acta Pharmacol. Toxicol.*, 40 (1977) 145.
11 C. T. Dollery, D. S. Davies, G. H. Draffan, H. J. Dargie, C. R. Dean, J. L. Reid, R. A. Clare and S. Murray, *Clin. Pharmacol. Ther.*, 19 (1976) 11.
12 B. Kolb, M. Linder and B. Kempken, *Appl. Chromatogr.*, 21E (1974) 1.
13 M. J. Hartigan, J. E. Purcell, M. Novotný, M. L. McConnell and M. L. Lee, *J. Chromatogr.*, 99 (1974) 339.
14 P. B. M. W. M. Timmermans, P. A. Van Zwieten and W. N. Speckamp, *Rec. Trav. Chim. Pays-Bas*, in press.
15 P. B. M. W. M. Timmermans, *Ph.D. Thesis*, University of Amsterdam, Amsterdam, 1976.