Journal of Chromatography, 613 (1993) 121–126
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6671

Determination of propranolol, labetalol and clenbuterol in rat brain by high-performance liquid chromatography

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(First received July 2nd, 1992; revised manuscript received November 16th, 1992)

ABSTRACT

A rapid and simple HPLC method for the measurement of adrenergic drugs (propranolol, labetalol and clenbuterol) in rat brain is described. This method was applied to establish if these drugs can pass the blood–brain barrier in prenatal or early post-natal life. The chromatography was performed using a C_{18} column and a phosphate buffer (pH 3)–acetonitrile (65:35, v/v) mixture. After homogenization of the brain tissue in perchloric acid, the supernatant was buffered at pH 9 and extracted with diethyl ether, followed by back-extraction in sulphuric acid. Recoveries of between 80 and 100% were achieved. The method was found to be accurate (100%) and precise (coefficient of variation around 10%). All three drugs were readily detected in the brain of neonatal rats after peripheral administration. In addition, we demonstrated the presence of propranolol in the fetal brain after maternal administration.

INTRODUCTION

It has been suggested that treatment during pregnancy or post-natal development with β-adrenergic blocking agents influences the adult setting of the central neurotransmitter systems [1–3]. However, a thorough investigation of the direct pharmacological effects of the treatment and the presence of the drugs in the brain is often neglected. If an experiment is carried out in the rat to study the effects of β-blockers on the developing brain it has to be established that the blocker can cross the blood–brain barrier and enter the brain in sufficient amount to block the central β-receptors.

Therefore we developed a simple and rapid HPLC method for measuring β-adrenergic antagonists and other drugs acting on adrenergic receptors in brain areas. Most methods describe the assay of β-adrenergic agents in plasma or urine [4–8]. Methods described for measurement in brain areas are not so practical as they include the use of benzene as extraction fluid [9] or gas chromatography [10] as assay method, or include the use of labelled (^{14}C or ^{3}H) β-adrenergic blocking agents [11,12]. An HPLC method for measuring β-adrenergic drugs in rat tissue has been described, but the recovery obtained after the extraction was low (25% for brain tissue) [8].

EXPERIMENTAL

Chemicals

Propranolol and labetalol were obtained from Sigma (Brunschwig Chemie, Amsterdam, Netherlands) and clenbuterol from Dopharma (Raamsdonkveer, Netherlands). Acetonitrile (Fisons, Rotterdam, Netherlands) was of HPLC–UV grade. The other chemicals and solvents, such as diethyl ether (Janssen Chimica, Tilburg, Netherlands), triethylamine, sulphuric acid, per-

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0378-4347/93/$06.00

chloric acid, sodium dihydrogenphosphate, sodium hydroxide (Merck, Amsterdam, Netherlands), sodium hydrogencarbonate, potassium carbonate and phosphoric acid (Baker, Deventer, Netherlands), were of analytical-reagent grade.

All aqueous solutions were prepared by using water obtained from a Milli-Q apparatus (Millipore, Etten-Leur, Netherlands).

The carbonate buffer pH 9.0 was prepared by dissolving 90 g of sodium carbonate and 32 g of potassium carbonate in a volume of 1000 ml of water.

Standard solutions

The standard stock solution of drugs was prepared by dissolving approximately 30 mg in 100 ml of water. The solution was divided into 0.5-ml portions and stored at −20°C for no longer than 6 months. These portions were diluted for further use in 0.2 *M* sulphuric acid.

The internal standard (I.S.) propranolol, used for clenbuterol and labetalol assays, was diluted to 1.48 μg/ml. The I.S. clenbuterol, used for the propranolol assay, was diluted to 31.4 μg/ml.

HPLC procedure.

The HPLC system was an HP 1090 (Hewlett-Packard, Amstelveen, Netherlands), equipped with an internal column oven (40°C), an autosampler and an internal UV filter detector (230 nm). The UV detector was connected to a computer in which the Baseline 810 (Dynamic Solution, Millipore, Etten-Leur, Netherlands) program was installed. The analytical column (250 × 4.6 mm I.D.) and the guard column (20 × 4.6 mm I.D.) were prepacked with LC-18-DB 5 μm (Supelchem, Leusden, Netherlands).

The mobile phase consisted of a mixture of acetonitrile–0.05 *M* sodium dihydrogenphosphate–triethylamine (35:65:0.1, v/v) and was adjusted to pH 3.0 with orthophosphoric acid. Before use the mobile phase was filtered through a Millipore 0.45-μm filter, type HA (Millipore), and during measurement it was continuously degassed with helium. The flow-rate was set at 1 ml/min.

Materials

Brain tissue for the recovery and validation experiments was obtained from adult male Wistar rats (Harlan CPB, Zeist, Netherlands). After decapitation, parts of the cerebral cortex were quickly dissected, frozen on dry ice and stored at −80°C.

This method was applied to the determination of adrenergic drugs in brain tissue of rats at different ages. Male Wistar rats, born in our animal house, were treated twice daily from post-natal day (PN) 1 to PN10 with propranolol [15 mg/kg subcutaneously (s.c.)] [1], labetalol (10 mg/kg s.c.) [13] or clenbuterol (2.5 mg/kg s.c.) [14]. The rat pups were decapitated on PN10 1.5 h after the last injection, and the occipital cortex or hippocampus was quickly dissected and frozen on dry ice. Another treatment was from gestational day (GD) 11 to GD21 with propranolol (40–50 mg/kg/day in drinking water) [15]. On GD21 the mothers were decapitated, the fetuses were taken out and half fetal brains were rapidly frozen. The tissue was stored at −80°C until it was used in the assay procedure.

Procedure

The frozen brain tissue was weighed and homogenized in four volumes of 0.4 *M* perchloric acid (PCA), using a motor-driven PTFE/glass homogenizer (Tamson, Zoetermeer, Netherlands) at 1400 rpm. The final tissue concentration was 25 mg/ml PCA. To each millilitre of PCA 40 μl of internal standard (I.S.) were added, which resulted in a concentration in the homogenate of 59 ng/ml for propranolol and 1256 ng/ml for clenbuterol. After centrifugation at 3000 *g* for 15 min, 1 ml of supernatant was transferred to a 10-ml polypropylene (PPN) tube. A 10-μl aliquot of 10 *M* sodium hydroxide and 350 μl of the carbonate buffer were added and vortexed for 10 s. After adding 8 ml of diethyl ether, the mixture was mechanically shaken for 45 min and centrifuged at 2000 *g* for 8 min. The diethyl ether layer was transferred to a clean PPN tube and 200 μl of 0.2 *M* sulphuric acid were added. The mixture was mechanically shaken for 15 min and centrifuged at 2000 *g* for 8 min. The diethyl ether layer

was discarded and the tube containing the acid layer was placed in a water bath at 45°C for about 1 h to evaporate the remaining traces of diethyl ether. A 50-μl aliquot of the acid layer was injected onto the column. Tissue standards were prepared by adding standard solutions to the homogenate of control tissue before centrifugation and treating and analysing them at the same time as samples.

RESULTS AND DISCUSSION

Chromatography and extraction

The HPLC method was modified from the method described by Dionisotti *et al.* [5]. The addition of triethylamine to the acetonitrile–phosphate mixture was necessary to avoid peak tailing. In Fig. 1 representative chromatograms of a standard and a control are shown.

The extraction procedure was a modification of that described by Winkler *et al.* [8]. In this method 10 *M* sodium hydroxide was added to the supernatant (0.4 *M* PCA) to make it alkaline (pH > 12) and ready for extraction. For the extraction of propranolol and clenbuterol this high pH was no problem, but labetalol was not extractable. For all compounds the recovery was at least 80% with the supernatant at pH 9.0. Therefore we neutralized the PCA with a smaller amount of 10 *M* sodium hydroxide (10 μl instead of 60 μl) and then buffered the supernatant at pH 9.0 with the carbonate buffer [7].

As organic phase, we tried hexane, diethyl ether [4,5] and a mixture of propanol–heptane (20:80) [8]. The extraction with hexane and the propanol–heptane mixture gave a low recovery (55–65%) and with hexane labetalol was not extractable at all. The use of diethyl ether caused a problem because traces of diethyl ether were absorbed by the acid layer during the back-extraction step. This resulted in a distorted peak shape and therefore irreproducible results. After placing the tube containing the acid layer in a water bath to remove the traces of diethyl ether this problem was solved and recoveries of at least 80% were obtained.

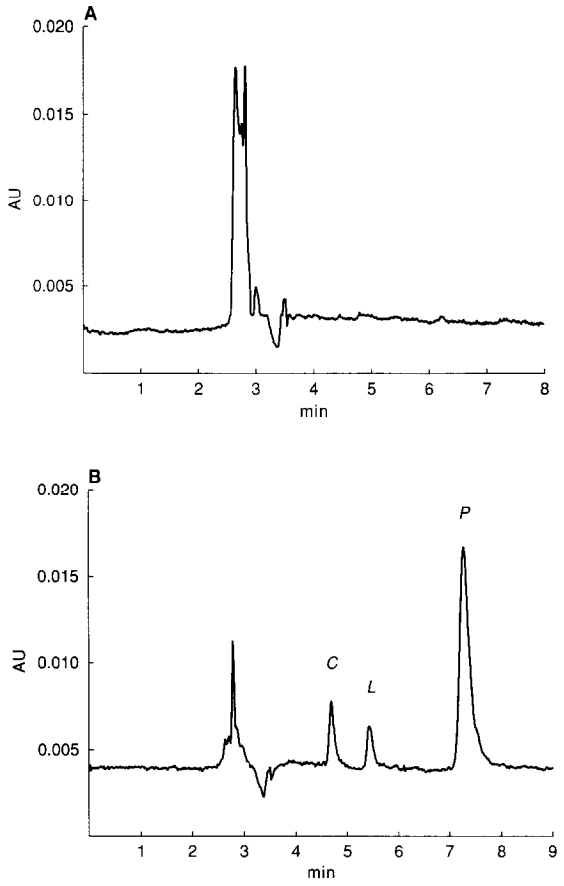

Fig. 1. (A) Chromatogram obtained from cortical tissue of a control rat. (B) Chromatogram obtained from the same tissue spiked with 1480 ng/ml propranolol (P), 628 ng/ml clenbuterol (C) and 730 ng/ml labetalol (L). HPLC conditions are described in the text.

Linearity and accuracy

Linearity and accuracy were determined by spiking control brain tissue with four different concentrations and treating and analysing them as samples. The linearity of the calibration curve was calculated with linear regression and was for the three components higher than 0.9991. The results are listed in Table I. The accuracy was the ratio of the measured (calculated) concentration compared with the added concentration. For all three components the accuracy was between 92 and 101%.

We used the ratio between the peak height of the I.S. and the drug measured for the calculation

of the linearity, accuracy, recovery and precision. This gave no improvement. Therefore we did not use the I.S. for the results in Table I.

Recovery, precision and detection limit

The recovery and within-day precision, expressed as the coefficient of variation (C.V.), are listed in Table I. The C.V. is calculated by the formula C.V. = standard deviation/mean of the recoveries × 100%. Only at the lowest concentration did the recovery deviate and the precision (C.V.) become higher. This is because these concentrations are 2–4 times the detection limit. The theoretical detection limit is calculated on the basis of a signal-to-noise ratio of 3. This is done in chromatograms from standards injected without extraction. For experimental samples this value is higher and equals about the lowest concentration tested for the drugs (Table I).

Winkler *et al.* [8] found a negative relation between recovery and tissue concentration in the homogenate. To test this, brain tissue was homogenized in different tissue concentrations (25 mg/ml, 50 mg/ml and 100 mg/ml PCA), and different concentrations of propranolol, labetalol and clenbuterol were added. These homogenates were extracted and analysed as described above. As the 100% value we took the concentrations used for the recovery in 0.2 *M* sulphuric acid and injected them without extraction onto the column (unextracted). The results are shown in Fig. 2. For propranolol and labetalol the tissue concentration had a negative influence on the recovery, whereas the recovery of clenbuterol was not affected by the different tissue concentrations.

TABLE I

VALIDATION OF THE DESCRIBED EXTRACTION AND CHROMATOGRAPHY OF PROPRANOLOL, CLENBUTEROL AND LABETALOL

Added (ng/ml)	Found (ng/ml)	Accuracy (%)	Recovery (%)	C.V.	*n*
Propranolol					
1480	1490	100.7	87.6	4.8	6
590	580	98.3	81.5	5.5	7
148	148	100.0	84.3	10.9	10
74	71	95.9	71.1	21.9	4
r = 0.9999					
Detection limit (DL) = 22 ng/ml					
Clenbuterol					
1570	1550	98.7	108.9	4.6	6
630	630	100.0	103.1	6.9	7
157	146	93.0	110.8	12.8	10
79	77	97.4	99.3	24.8	4
r = 0.9997					
DL = 33 ng/ml					
Labetalol					
1830	1810	98.9	88.1	8.2	6
730	720	98.6	80.5	6.1	7
183	170	92.9	70.8	15.5	10
92	108	117.4	93.3	26.1	4
r = 0.9993					
DL = 33 ng/ml					

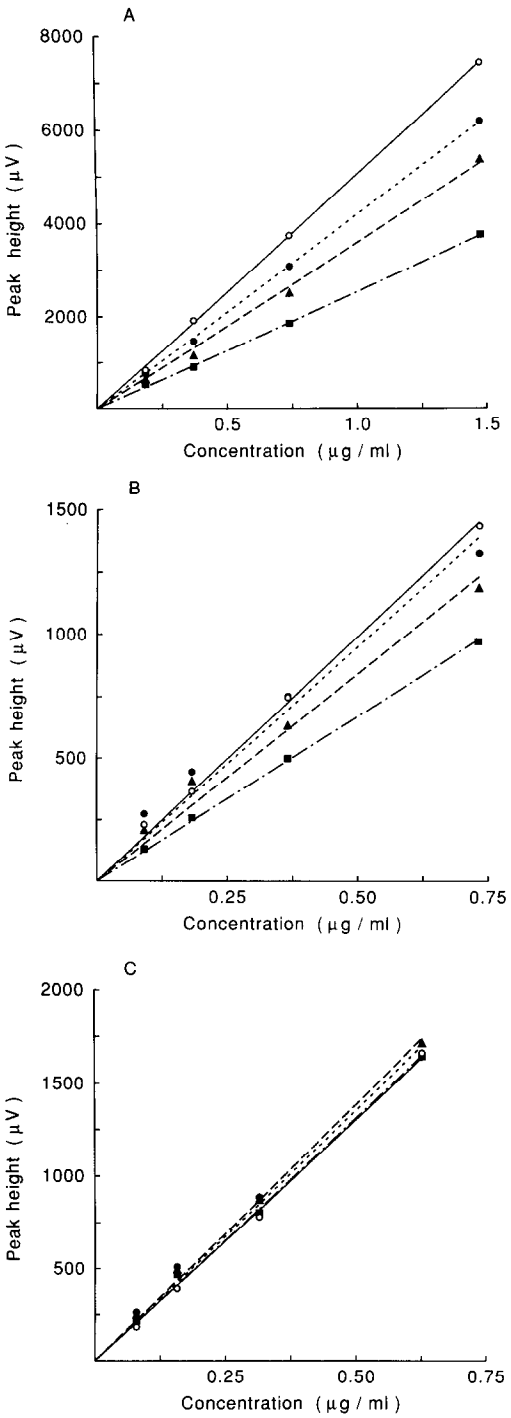

Fig. 2. Influence of the tissue concentration in the homogenate on the recovery for propranolol (A), labetalol (B) and clenbuterol (C). The peak height is given in μV. One volt corresponds to two absorbance units (AU). ● - - - ● = 25 mg/ml; ▲ - - - ▲ = 50 mg/ml; ■— - —■ = 100 mg/ml; ○ ——— ○ = unextracted.

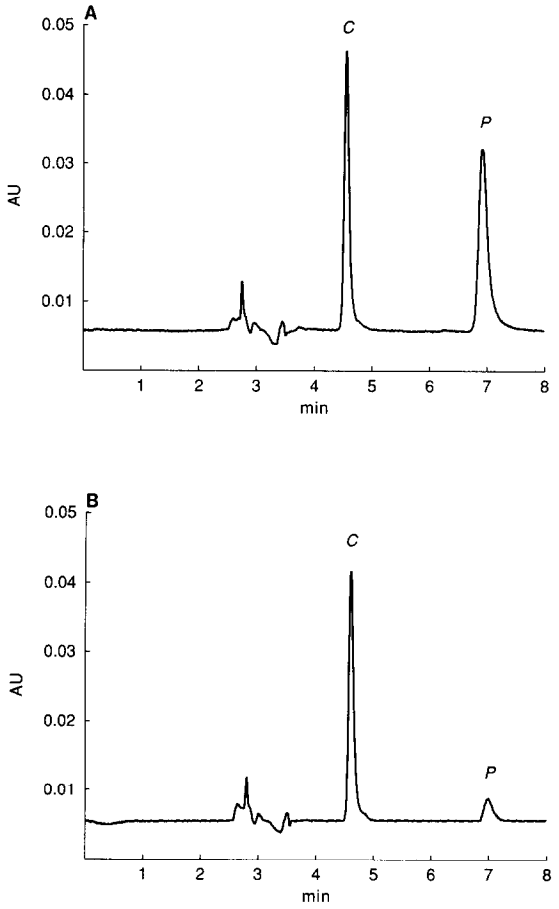

Fig. 3. (A) Chromatogram obtained from cortical tissue of a rat treated with propranol (15 mg/kg, twice daily) from post-natal days (PN) 1 to 10, decapitated 1.5 h after the last injection on PN10. The concentration of propranol (P) is 16 μg per g of tissue. (B) Chromatogram obtained from half a brain of a rat fetus, decapitated on gestational day (GD) 21. The mother was treated orally with 50 mg/day propranolol. The concentration of propranolol (P) is 2.5 μg per g of tissue. Clenbuterol (C) was added as internal standard at a concentration of 314 ng per injection.

Determination of the brain concentration

To apply the described method, brain concentrations of the β-adrenergic drugs were measured after different treatments in rats. Examples of the chromatograms obtained after the extraction and chromatography are shown in Figs. 3 and 4. The treatment, the dose and the calculated concentration are listed in the figure captions. Brain concentrations after s.c. injections on PN10 were easily measurable, suggesting that substantial

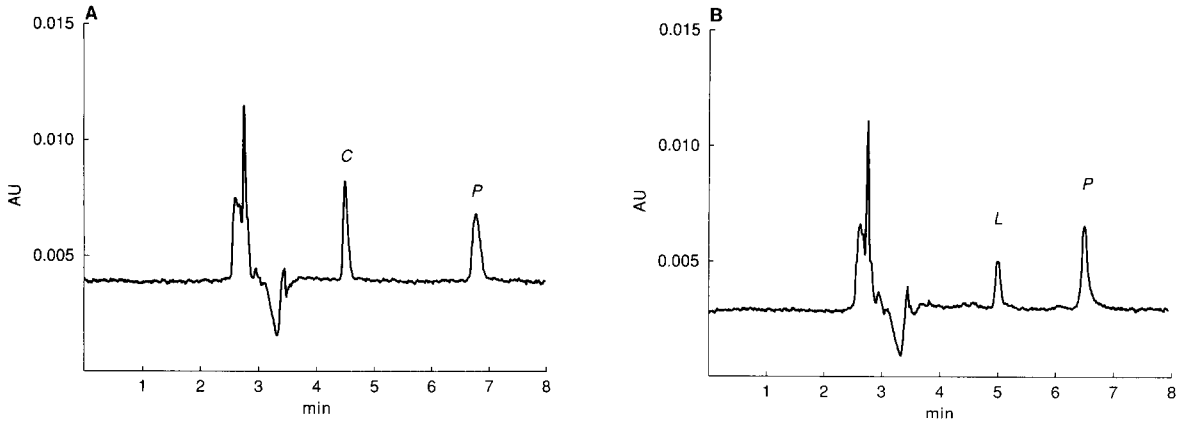

Fig. 4. (A) Chromatogram obtained from hippocampal tissue of a rat treated with clenbuterol (2.5 mg/kg, twice daily) from PN1 to PN10, decapitated 1.5 h after the last injection on PN10. The concentration of clenbuterol (C) is 5.0 μg per g of tissue. (B) Chromatogram obtained from cortical tissue of a rat treated with labetalol (10 mg/kg, twice daily) from PN1 to PN10, decapitated 1.5 h after the last injection on PN10. The concentration of labetalol (L) is 3.2 μg per g of tissue. Propranolol (P) was added as internal standard at a concentration of 14.8 ng per injection.

amounts of the β-adrenergic drug reach the neonatal brain. Even treatment with a considerably lower dose should therefore be measurable. Prenatal maternal oral ingestion of propranolol resulted in lower but easily detectable brain concentrations. If fluorimetric detection could be used instead of UV, measurement of even lower concentrations would be possible.

CONCLUSION

In this paper a method for measuring adrenergic drugs in rat brain areas is described. As shown in Table I, the method is precise and accurate for the β-antagonist propranolol, the β/α_1-antagonist labetalol and the β-agonist clenbuterol. The recovery is reproducible (precision of 10%) and high (at least 80%) with the extraction method used. In practice, the method is easy to use to establish whether β-adrenergic drugs can pass the blood–brain barrier after administration in rats (see Figs. 2–4) [13].

The extraction method is also suitable for other adrenergic drugs such as the α_2-antagonist yohimbine, the α_2-agonist clonidine and α_1-antagonist prazosin (data not shown).

REFERENCES

1 B. H. W. Erdtsieck-Ernste, M. G. P. Feenstra, G. J. Boer and H. van Galen, *Brain Res. Bull.*, 26 (1991) 731.
2 L. A. Hilakivi, T. Taira, E. MacDonald, L. Tuomisto and K. Hellevuo, *Psychopharmacology*, 96 (1988) 353.
3 Z. Speiser, A. Shved and S. Gitter, *Psychopharmacology*, 79 (1983) 148.
4 S. A. Qureshi and H. S. Buttar, *J. Chromatogr.*, 431 (1988) 465.
5 S. Dionisotti, F. Bamonte and A. Monopoli, *J. Chromatogr.*, 530 (1990) 458.
6 F. F. T. Ververs, H. G. Schaefer, J. F. Lefevre, L. M. Lopez and H. Derendorf, *J. Pharm. Biomed. Anal.*, 8 (1990) 535.
7 B. Oosterhuis, M. van den Berg and C.J. van Boxtel, *J. Chromatogr.*, 226 (1981) 259.
8 H. Winkler, W. Ried and B. Lemmer, *J. Chromatogr.*, 228 (1982) 223.
9 H. L. Garvey and N. Ram, *J. Pharmacol. Exp. Ther.*, 194 (1975) 220.
10 G. Bianchetti, J. L. Elghozi, R. Gomeni, P. Meyer and P. L. Morselli, *J. Pharmacol. Exp. Ther.*, 214 (1980) 682.
11 J. F. M. Smits and H. A. J. Struyker-Boudier, *J. Pharmacol. Exp. Ther.*, 209 (1979) 317.
12 H. S. Buttar, J. H. Moffatt and C. Bura, *J. Toxicol Environment. Health*, 24 (1988) 1.
13 E. B. H. W. Erdtsieck-Ernste, M. G. P. Feenstra, M. H. A. Botterblom, J. de Barrios and G. J. Boer, *Br. J. Pharmacol.*, 105 (1992) 37.
14 E. B. H. W . Erdtsieck-Ernste, M. G. P. Feenstra, M. Botterblom and G. J. Boer, *Dev. Brain Res.*, in press.
15 E. B. H. W. Erdtsieck-Ernste, M. G. P. Feenstra, M. Botterblom and G. J. Boer, *Neurochem. Int.*, in press.