Journal of Chromatography, 231 (1982) 255—264
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1333

SIMULTANEOUS DETERMINATION OF MORPHINE AND MONOAMINE TRANSMITTERS IN A SINGLE MOUSE BRAIN

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(First received January 29th, 1982; revised manuscript received April 17th, 1982)

SUMMARY

A simple procedure for the simultaneous determination of morphine and monoamine transmitters was developed. The procedure consisted of (1) *n*-butanol extraction and (2) separation and quantitative determination by means of high-performance liquid chromatography combined with electrochemical detection. The maximum intracerebral concentration (210 ± 35 ng/g wet tissue) of morphine was detected 30 min after intramuscular injection (10 mg/kg), which agreed with previous research. Noradrenaline was significantly decreased by morphine injection, while dopamine and 5-hydroxytryptamine were unchanged. However, 3-methoxytyramine, a metabolite of dopamine, was increased, suggesting that the drug increased the turnover rate of dopamine. The procedure used revealed a direct correlation between pharmacokinetics (e.g., distribution of morphine) and pharmacodynamics (e.g. changes of monoamine concentrations) of the drug in vivo.

INTRODUCTION

Research in modern experimental pharmacology involves two major subdivisions — pharmacokinetics and pharmacodynamics [1]. Pharmacodynamics is concerned with biochemical and physiological effects of drugs and their mechanisms of actions. The effect of drug is related to the concentration of drug at its site of action. On the other hand, pharmacokinetics, which deals with the absorption, distribution, biotransformation and excretion of drugs, determines the concentration of a drug at its site of action after it is administered.

Some drugs, as one of their actions, change the concentrations of monoamine transmitters in the brain. It is important to measure simultaneously concentrations of the administered drug and the transmitter substances which

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are affected by the drug in the same sample, because a simultaneous determination will determine whether there is a direct correlation between pharmacokinetics and pharmacodynamics in vivo. Previously, the concentrations of catecholamines and potent inhibitors of aromatic-L-amino acid decarboxylase, α-difluoromethyldopa and α-monofluoromethyldopa were measured in the same sample of mouse brain [2].

While morphine is known to affect the monoaminergic nervous systems, there is no general agreement as to its site of action or underlying mechanism [3, 4]. If, however, a brain assay procedure for the simultaneous determination of morphine and amine transmitters were developed, then it is possible that the precise effect of the drug on monoaminergic systems will be better understood.

Numerous investigators have reported the usefulness of high-performance liquid chromatography combined with electrochemical detection (HPLC—ElCD) for the determination of monoamine transmitters, precursor amino acids and metabolites in biological samples [5—11]. This detector has been reported to be sensitive for substances having phenolic hydroxy group(s) in its molecule [12]. Morphine is a drug which has a phenolic hydroxy group and some reports have demonstrated procedures for the determination of morphine concentrations using HPLC—ElCD [13, 14]. These findings suggest that the detection system should be able to determine morphine and monoamine transmitters simultaneously in biological samples if the appropriate extraction and chromatographic separation procedures are developed. The present study demonstrates a simple procedure for the determination of morphine together with noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), tyrosine, tryptophan and 3-methoxytyramine (3-MT) in a single mouse brain.

MATERIALS AND METHODS

Apparatus

The liquid chromatography system was assembled using commercially available components including a pump (high-pressure precision pump, A-30-S, Eldex Labs., Menlo Park, CA, U.S.A.), six-port injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) and electrochemical detector (VMD-101, Yanagimoto, Kyoto, Japan). The working electrode was of glassy carbon. The chromatographic column was an Ultrasphere-ODS (average particle size, 5 μm) prepacked in a stainless-steel column, 25 cm × 4.6 mm I.D. (Altex Scientific, Berkeley, CA, U.S.A.). The chromatography was performed at a constant flow-rate of 0.85 ml/min using a 0.05 *M* sodium citrate–citric acid buffer (pH 4.25) containing 1% tetrahydrofuran. The electrode-applied potential was set at 725 mV vs. the Ag/AgCl reference electrode.

Reagents

Reagent-grade chemicals for extraction and chromatography included hydrochloric acid, disodium ethylenediaminetetraacetate, sodium citrate, citric acid, *n*-butanol, *n*-heptane and tetrahydrofuran, and were used without further purification. Morphine sulfate was purchased from Merck (Rahway,

NJ, U.S.A.), and authentic standards for monoamine-related substances from Sigma (St. Louis, MO, U.S.A.). These included noradrenaline·HCl, dopamine·HCl, 5-hydroxytryptamine·HCl, 3-methoxytyramine·HCl, tyrosine and tryptophan. Each substance was separately dissolved in 0.1 *N* hydrochloric acid at a concentration of 1.0 mg/ml as free base. The internal standard was 3,4-dihydroxybenzylamine (Sigma), which was also dissolved in 0.1 *N* hydrochloric acid at a concentration of 1 μg/ml. These standard substances were freshly prepared each month and were stable for over one month when stocked in a refrigerator at 4°C. The working standard solution, including authentic substances, was made from the stock solutions each day of the assay.

Animals

Male Swiss-Webster mice were used. Animals were housed in an air-conditioned room with a 12-h light—dark cycle for a minimum of one week prior to the experiment. Morphine was intramuscularly administered at a dose of 10 mg/kg. The animals were sacrificed 10, 30, 60 or 120 min after the injection by decapitation, which was performed between 10:00 a.m. and 12:00 noon to minimize circadian fluctuation in monoamine levels. After decapitation, the brain was removed as quickly as possible and stored on dry ice until the assay was carried out. The assay was, in general, completed within one week after sacrifice. The control animals received saline injection.

Extraction procedure

Each brain was weighed and transferred to a glass tube containing 500 μl of 0.1 *N* hydrochloric acid, 20 μl of 0.1 *M* EDTA and 500 ng of the internal standard. The brain was homogenized in an homogenizer (Tissuemizer, Janke and Kunkel, G.F.R.) and then transferred to a screw-capped vial (15 ml) which contained 10 ml of *n*-butanol and 4 g of solid sodium chloride. The vial was then shaken on a reciprocal shaker for 60 min. After centrifugation at 5000 rpm for 10 min, 9 ml of the butanol layer were transferred to another vial (50 ml) containing 200 μl of 0.1 *N* hydrochloric acid and 18 ml of heptane. The vial was shaken again on the shaker for 10 min and centrifuged at 5000 rpm for 5 min. A 20-μl volume of the acid aqueous layer was taken directly from the bottom of vial using a microsyringe (710 SNR, Hamilton, Reno, NV, U.S.A.) and injected onto the column.

Calibration

For calibration, authentic standard substances were taken through the entire procedure of extraction. A 400-μl volume of the working standard solution (which contained 400 ng each of NA and 5-HT; 800 ng of DA; 3 μg of tryptophan; 6 μg of tyrosine; and 20 ng of 3-MT) was pipetted into a screw-capped vial containing the same chemicals as described under *Extraction procedure*. The various concentrations of standard substances were derived from those in mouse whole brain. Different amounts of morphine (1—200 ng/tube) were added to the vials and the extraction was then carried out exactly as described in the procedure section. Quantitative determinations of monoamine transmitters and precursor amino acids were based on comparison of relative peak heights to the internal standard.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

Previous determinations of morphine concentration by HPLC—ElCD [13, 14] used different extraction methods. In neither case were the extraction procedures effective for simultaneous determination of morphine and monoamine transmitters in the same sample. Although a preliminary experiment showed that a *n*-butanol—chloroform mixture [15] was useful for the extraction of morphine and indoleamine, it was not effective for the assay of catecholamines. We recently demonstrated a simple procedure for the determination of monoamine transmitters and their precursor amino acids and metabolites in the same sample of mouse whole brain [10]. In that experiment, an organic solvent extraction procedure [16] was used with some modification according to sample size. The procedure consisted of (1) primary extraction of monoamines into *n*-butanol under acidic condition and (2) re-extraction of the substances from the butanol layer into hydrochloric acid by decreasing the solubility of the substances by adding heptane. This procedure may not be specific for the extraction of substances related to monoamine metabolism. The electrochemical detector has been reported to be relatively specific for substances having a phenolic hydroxy group. Since morphine as well as monoamine transmitters have such hydroxy group(s), they can be expected to be specifically measured by the electrochemical detection. On the other hand, the improvement in packing materials for liquid chromatography produces a column which has a high theoretical plate. This fact makes it easier to separate individual substances from a complex matrix of biological origin. If both a specific detector and high efficiency column are available for chromatography, a non-specific extraction procedure can be favorable for a simultaneous determination. Hence, we decided to use butanol extraction procedure in this study.

The selectivity of the electrochemical detector is inversely related to the applied oxidation potential [12]. Fig. 1 shows the change in response current against applied potential (current–potential curve). Morphine, which has one phenolic hydroxy group on position 3, initiated a response current at 425 mV vs. the Ag/AgCl reference electrode. This value was slightly different from that of a previous report [13]. The difference may be due to the character of individual electrodes. The current—potential curve observed for morphine is probably due to one electron oxidation of the phenolic hydroxy group. Codeine, which has no hydroxy group on position 3, did not respond with the same applied voltage. Monoamine transmitters generated their own response current at lower applied potentials than the drug, which were 200 mV for catecholamines and 300 mV for indoleamine. Similar current—potential curves have been reported for catecholamines [11]. On the other hand, the precursor amino acids, tyrosine and tryptophan, needed higher potentials to generate a current. 3-Methoxytyramine, an intermediary metabolite of DA, responded at almost the same applied potential as morphine (not shown in Fig. 1, see ref. 10). For the assay of monoamine transmitters alone, a value under 600 mV is recommended because of low background noise [10]. Although increasing the applied potential produces a greater response current

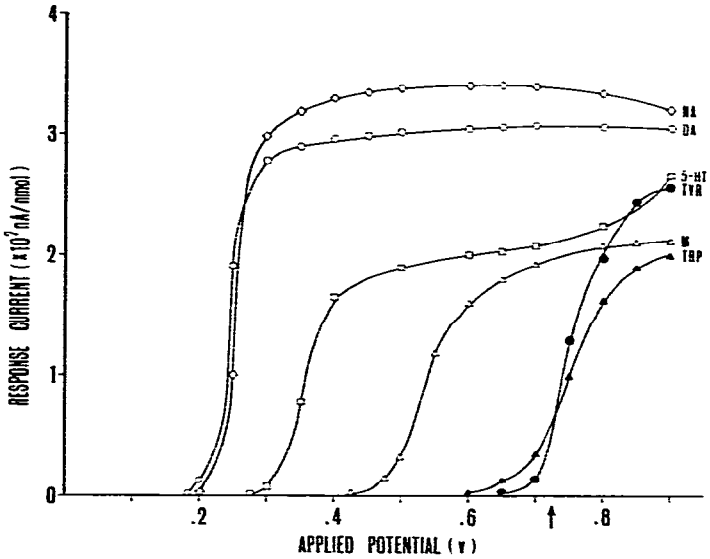

Fig. 1. Current—potential curves for noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), tyrosine (TYR), tryptophan (TRP) and morphine (M). The arrow shows the applied potential used in the present study (725 mV).

for all substances (Fig. 1), higher potentials also make greater background current and noise levels, which decrease the specificity of the detector [12]. The samples obtained by butanol extraction showed no disadvantage in detector specificity for morphine even when the applied potential was increased from 600 mV to 725 mV. This also made it possible to detect precursor amino acids in brain samples (Figs. 1 and 2). Applied potentials above 750 mV produced an unknown peak which interfered with the determination of morphine. The applied potential was therefore selected at 725 mV vs. the reference electrode to detect simultaneously the drug and monoamine transmitters and precursors, since morphine is known to affect both of them [3, 4, 17, 18].

The most important contribution of this study was the selection of a mobile phase that could effectively separate morphine from biogenic substances. Morphine is presumed to have a long retention time on a reversed-phase column because of its physiochemical properties. It is generally thought that decreasing the polarity of the mobile phase results in shortened retention time of polar substances in this type of column. A previous determination of morphine by means of HPLC—ElCD used a high concentration of methanol (85%) in buffer for the separation [14]. However, a high organic concentration in the mobile phase is not suited for electrochemical reactions [12]. In a preliminary experiment, only 20% methanol in buffer was found to decrease electrode efficiency by about 30% of non-methanol buffer for morphine, as well as for monoamine transmitters. This means that the mobile phase containing a high concentration of methanol cannot be used, at least in the present detection system. Tetrahydrofuran is a strong non-polar solvent and was used for adjusting the polarity of the mobile phase. This solvent shortens the retention time of amines about ten-fold as compared to methanol. In a

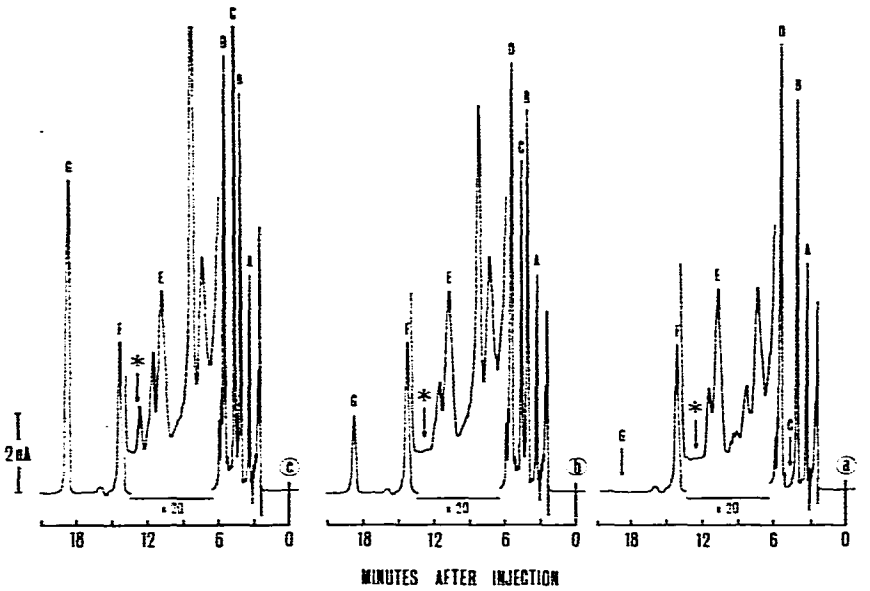

Fig. 2. Chromatograms obtained with different applied potentials: a, 600; b, 725; and c, 750 mV. Asterisks show the retention times for morphine. Peaks: A = noradrenaline; B = 3,4-dihydroxybenzylamine (internal standard); C = tyrosine; D = dopamine; E = 3-methoxytyramine; F = 5-hydroxytryptamine; G = tryptophan.

previous report [10], we successfully used 1% tetrahydrofuran for the separation of substances related to monoamine metabolism. This substance was also effective for the separation of morphine from other biogenic substances and was therefore used as an organic solvent for adjusting the polarity of the mobile phase.

Also, we found that the pH value of the mobile phase critically influenced the retention time of morphine, while ionic strength did not. Decreasing the pH value resulted in a shorter retention time for morphine (Fig. 3). The retention time converged with that of 3-MT when the pH was decreased to 3.0. On the other hand, a pH value of 5.5 yielded a different retention time for morphine from those of 5-HT and tryptophan. Although pH 5.5 was effective for the separation of these substances, it was not appropriate for morphine determination because the peak of morphine showed a significant tailing that was probably due to non-linear absorption of the drug on the column. Furthermore, DA and tyrosine had almost the same retention time in this pH condition, which made it impossible to separate and detect both substances (Fig. 3). Thus, we chose to use a pH of 4.25 in the present experiment. The theoretical plate number was estimated to be about 10,000 per meter for morphine and was greater for monoamine transmitters.

The present electrochemical detector provided linear responses within a wide range of doses from 500 pg to 50 ng (Fig. 4). This made it possible to calculate the concentration by a simple measurement of peak height for morphine as well as monoamine-related substances.

The recovery rates of substances using the *n*-butanol extraction procedure were determined in the presence of tissue samples (Table I). The recovery of

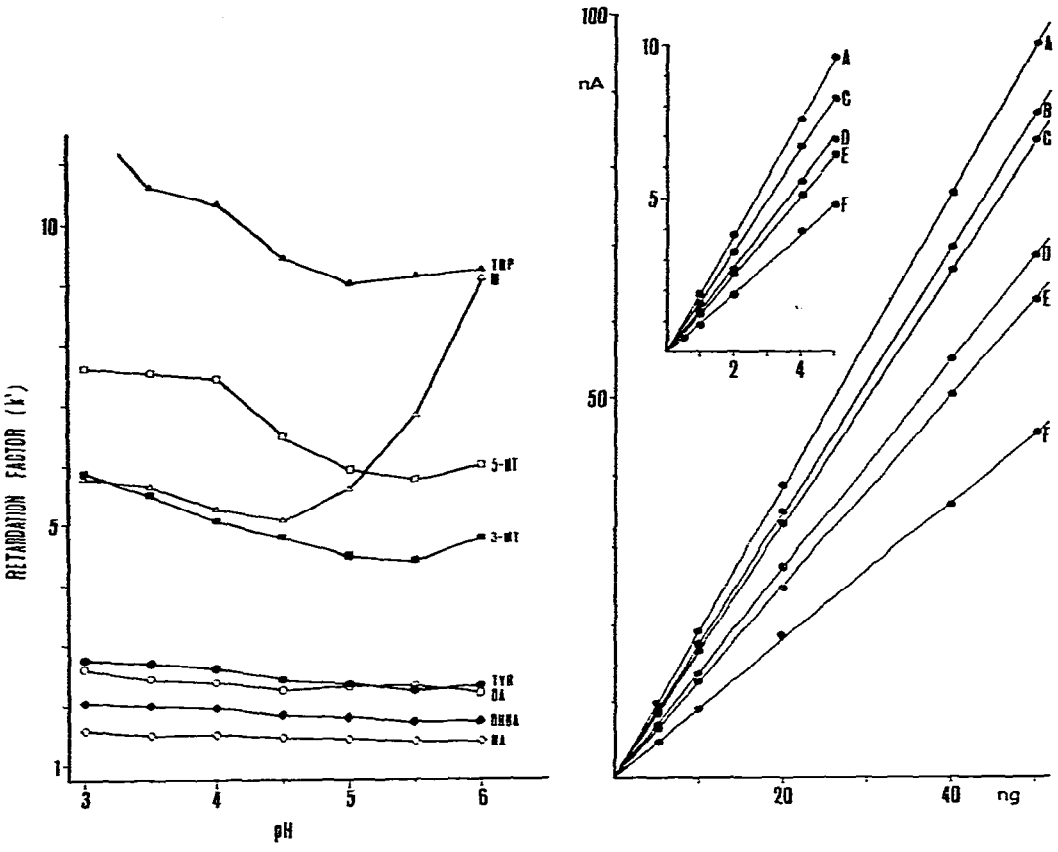

Fig. 3. Effect of pH on the retention of monoamine-related substances and morphine. 3-MT = 3-Methoxytyramine; DHBA = 3,4-dihydroxybenzylamine; other abbreviations as in Fig. 1.

Fig. 4. Standard curves of monoamine transmitters and morphine. A = Noradrenaline; B = 3,4-dihydroxybenzylamine; C = dopamine; D = 5-hydroxytryptamine; E = 3-methoxytyramine; F = morphine.

TABLE I

RECOVERY RATES, DETECTION LIMITS AND RETENTION TIMES FOR THE SUBSTANCES EXAMINED

Substance	Recovery rate* (%)	Detection limit (ng)	Retention time (min)
NA	60.0 ± 3.7	0.2	3.3
DA	64.7 ± 3.4	0.2	5.8
5-HT	54.6 ± 2.9	0.5	14.0
Tyrosine	56.7 ± 2.8	2	4.4
Tryptophan	39.2 ± 4.6	2	19.1
3-MT	51.8 ± 3.6	0.5	10.5
Morphine	72.1 ± 4.8	1	12.2

*The values represent means ± S.D. from 6 determinations.

morphine was over 70%. A preliminary experiment using a *n*-butanol—chloroform mixture showed a greater recovery of the drug than that of the present extraction procedure, while catecholamines were not extracted. These recoveries for morphine and monoamine transmitters were included in the estimation of tissue concentrations. The detection limits and retention times for the substances are also summarized in Table I.

Application to tissue sample

Fig. 5 shows a typical chromatogram for the sample obtained from an animal injected intramuscularly with 10 mg/kg morphine 30 min before sacrifice. The present combination of *n*-butanol extraction and HPLC—ElCD was effective for the simultaneous determination of morphine and substances related to monoamine metabolism (three monoamine transmitters, two precursor amine acids and one intermediary metabolite) in mouse whole brain. The identification of each peak was carried out by a previously reported procedure, in which both chromatographic and electrochemical behavior were compared with the standard substance [7]. Some unknown peaks were recorded on the chromatogram of the present sample. The peaks did not, however, interfere with determination of either morphine or monoamines. A previous report [19] demonstrated that HPLC—ElCD is effective for the determination of catecholamines and indoleamine in dissected regions of the hippocampus (which weighed about 20 mg). This means that the present procedure can be used for the simultaneous determination of regional concentrations of morphine and monoamine transmitters.

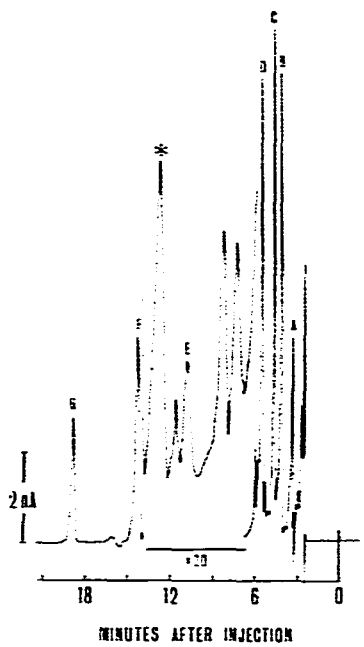

Fig. 5. Representative chromatogram obtained from the brain of animal injected intramuscularly with 10 mg/kg morphine 30 min before sacrifice. See text for the procedures of extraction and chromatography. Peaks as in Fig. 2. The asterisk shows the peak of morphine. The sensitivity under the bar was 20 times greater.

The changes in intracerebral concentrations of morphine and the substances related to monoamine metabolism were measured at different intervals after intramuscular injection of the drug (10 mg/kg). The time course of morphine concentration in the brain (Table II) agreed with previous findings [20]. The maximum value was observed 30 min after an injection. The drug was detectable after 2 h at about 50% of the maximum concentration.

TABLE II

CONCENTRATIONS OF MONOAMINE-RELATED SUBSTANCES AND MORPHINE IN MOUSE WHOLE BRAIN SAMPLE AFTER A 10 mg/kg INTRAMUSCULAR INJECTION

Values are expressed in ng/g wet tissue and means ± S.D. from 6 animals.

Substance	Control	Minutes after injection			
		10	30	60	120
Morphine	—	180 ± 19	210 ± 35	205 ± 15	136 ± 18
NA	468 ± 25	420 ± 54	382 ± 31*	409 ± 16*	417 ± 10*
DA	916 ± 51	999 ± 39	976 ± 44	967 ± 46	990 ± 71
5-HT	509 ± 31	527 ± 49	502 ± 85	518 ± 52	534 ± 36
Tyrosine	7335 ± 610	7404 ± 703	7245 ± 879	7277 ± 482	5544 ± 357*
Tryptophan	3732 ± 523	3733 ± 515	3700 ± 505	3387 ± 351	3148 ± 276
3-MT	25 ± 2	29 ± 5	38 ± 6*	39 ± 3*	35 ± 3*

*Significantly different from control group ($P < 0.01$).

Morphine has been reported to decrease intracerebral levels of catecholamines and indoleamine in both mouse and rat brain [3, 4]. The decreases are followed by increases in the levels of monoamine metabolites [3, 4]. These results suggest that morphine produces an increase in turnover rates of the monoamines. The present results agree, in general, with those of previous findings. Morphine significantly decreased ($P < 0.01$) the level of NA 30 min after an intramuscular injection, a time at which the maximum concentration of morphine was observed (Table II). On the other hand, the levels of DA and 5-HT were not affected by the injection.

Morphine has been also reported to affect the levles of precursor amino acids, tyrosine and tryptophan [17, 18]. The present results revealed a decrease of tyrosine when detected 120 min after injection, while tryptophan was not affected. A metabolite of DA, 3-MT, was significantly increased ($P < 0.01$) by morphine injection. This suggests that morphine increased the turnover rate of DA, confirming a previous report [21]. The procedure reported here does not directly measure the concentrations of metabolites of NA and 5-HT as well as other metabolites of DA. However, in previous studies [10, 19], we reported a procedure for the determination of those metabolites in mouse brain which used the organic layer remaining after the present hydrochloric acid re-extraction. This means that the proposed procedure can measure the concentrations of monoamine metabolites such as 3-methoxy-4-

hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindoleacetic acid in the sample.

The purpose of this study was to develop a procedure for the simultaneous determination of morphine and monoamine transmitters in a single brain. HPLC—ElCD combined with butanol extraction is a useful technique because the direct correlation between pharmacokinetics and pharmacodynamics of morphine can be uncovered in an in vivo preparation. A more detailed report of the biochemical effects of morphine on monoaminergic neuron systems will appear elsewhere [22]. The procedure may be also applied, with minor modification, to the determination of other drugs having phenolic hydroxy groups.

REFERENCES

1 S.E. Mayer, K.L. Melmon and A.G. Gilman, in A.G. Gilman, L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 1980, p. 1.
2 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41.
3 E.L. Way and F.-H. Shen, in D.H. Clouet (Editor), Narcotic drugs: Biochemical Pharmacology, Plenum Press, New York, 1971, p. 229.
4 E.T. Iwamoto and E.L. Way, in H.H. Loh and D.H. Ross (Editors), Neurochemical Mechanisms of Opiates and Endorphins, Raven Press, New York, 1979, p. 357.
5 S. Sasa and C.L. Blank, Anal. Chem., 49 (1977) 354.
6 L.J. Felice, J.D. Felice and P.T. Kissinger, J. Neurochem., 31 (1978) 1461.
7 D.D. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
8 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, J. Chromatogr., 162 (1979) 7.
9 E. Kempf and P. Mandel, Anal. Biochem., 112 (1981) 223.
10 K. Ishikawa and J.L. McGaugh, J. Chromatogr., 229 (1982) 35.
11 R.C. Causon, M.E. Carruthers and R. Rodnight, Anal. Biochem., 116 (1981) 223.
12 P.T. Kissinger, Anal. Chem., 48 (1977) 442A.
13 M.W. White, J. Chromatogr., 178 (1979) 229.
14 J.E. Wallace, S.C. Harris and M.W. Peek, Anal. Chem., 52 (1980) 1328.
15 H. Kupferberg, A. Burkhalter and L.E. Way, J. Pharmacol. Exp. Ther., 145 (1964) 247.
16 A.S. Welch and B.L. Welch, Anal. Biochem., 30 (1969) 161.
17 R.B. Messing, C. Flinchbauch and J.C. Waymire, Neuropharmacology, 17 (1978) 391.
18 A.A. Larson and A.E. Takemori, J. Pharmacol. Exp. Ther., 200 (1977) 216.
19 K. Ishikawa, T. Ott and J.L. McGaugh, Brain Res., 232 (1982) 222.
20 C. Cerletti, S.H. Keinath, R.J. Tallarida, M.M. Reidenberg and M.W. Adler, Substance Alcohol Actions/Misuse, 1 (1980) 65.
21 R. Papeschi, P. Theiss and A. Herz, Eur. J. Pharmacol., 34 (1975) 253.
22 K. Ishikawa, S. Shibanoki and J.L. McGaugh, in preparation.