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SIMULTANEOUS DETERMINATION OF BIOGENIC AMINES AND MORPHINE IN DISCRETE RAT BRAIN REGIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple and sensitive method has been developed for the simultaneous determination of norepinephrine, epinephrine, dopamine, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, and morphine in discrete rat brain regions by reversed-phase high-performance liquid chromatography with electrochemical detection. Perchloric acid extracts of the tissue were directly injected into the chromatographic system. Each of these compounds gave a linear response over the range of 20–160 ng/ml cerebellar homogenate (0.4–3.2 ng on column). Recoveries of these compounds, added to the homogenates, were complete when compared with standards dissolved in perchloric acid. The average between-run coefficients of variation for all these compounds were lower than 7.4% over the range of 20–160 ng/ml, and the within-run coefficients of variation at 20 ng/ml were lower than 8.7%. The present method has been applied to a study of the effects of intraperitoneal administration of morphine on biogenic amines in several discrete rat brain regions.

INTRODUCTION

There has been an exponential increase in the number of literature accounts of the use of high-performance liquid chromatography (HPLC) for neurochemical analysis of endogenous biogenic amines in biological samples[1–20]. A recent study of the pharmacokinetics and pharmacodynamics of morphine, necessitating the simultaneous measurement of biogenic amines and morphine in whole brain, also employed HPLC[21]. The availability of a method for the simultaneous determination of biogenic amines and morphine would prove useful in studies addressing the relation between morphine-induced changes in biogenic amine transmission and the behavio-

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ral effects of the drug. Changes in steady-state levels or turnover rates of biogenic amines in various brain regions after morphine administration could be interpreted in the light of the knowledge of the relative levels and rates of elimination of morphine in those regions.

Unfortunately, the method of Ishikawa *et al.*[21] who used HPLC with organic solvent extraction as a means of purification, not only gave poor chromatographic separation but also resulted in low recoveries for both biogenic amines and morphine. We describe here a modification of the method of Kim *et al.*[15], originally used for measuring biogenic amines in various brain regions, which allows a sensitive and specific simultaneous determination of biogenic amines and morphine by the use of HPLC with electrochemical detection (ED).

The present method has been applied to a study of the effects of intraperitoneal administration of morphine on the biogenic amine levels in several discrete regions of the rat brain. The levels of morphine in blood were also measured by HPLC at various time intervals after a single i.p. injection of graded doses of morphine.

EXPERIMENTAL

Materials

Norepinephrine bitartrate (NE) (No. A-9512), epinephrine (E) (No. E-4375), dopamine hydrochloride (DA) (No. H-8502), 5-hydroxytryptamine creatinine sulfate complex (5-HT) (No. H-7752), 5-hydroxyindoleacetic acid (5-HIAA) (No. H-8876) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) (D-7012) were purchased from Sigma (St. Louis, MO, U.S.A.), citric acid (B-10081), disodium hydrogen orthophosphate (No. 10249) and morphine sulfate (Lot No. 88675) were from British Drug Houses (Toronto, Canada), disodium ethylenediaminetetraacetic acid (EDTA) (S-311) and 70% perchloric acid (PCA) (ACS Reagent, A-229) were from Fisher (Fair Lawn, NJ, U.S.A.), sodium octyl sulfate (No. 10577) was from Eastman Kodak (Rochester, NY, U.S.A.) and methanol (HPLC Grade) was from Caledon Labs. (Georgetown, Canada). Water was deionized and glass distilled.

HPLC apparatus

HPLC determinations were performed with a Beckman (Irvine, CA, U.S.A.) Model 330 isocratic liquid chromatograph and a Model 110A Pump, a Bioanalytical Systems (West Lafayette, IN, U.S.A.) Model LC-4B amperometric detector and a Hewlett-Packard (Palo Alto, CA, U.S.A.) 3390A recording integrator. A glassy carbon working electrode was set at 0.8 V *vs.* an Ag/AgCl reference electrode. Sensitivity of the detector was maintained from 0.2 to 1.0 nA f.s., depending on the concentration of the substance to be measured. Separation was performed on a 250 mm × 4.6 mm I.D. Altex Ultrasphere ODS column (C_{18} reversed-phase, particle size 5 μm) preceded by a guard column (Guard-Pak, C_{18}, Waters Assoc., Milford, MA, U.S.A.) with isocratic elution.

Mobile phase

The mobile phase (pH 4.2) contained 50 m*M* citric acid, 100 m*M* disodium hydrogen orthophosphate, 0.38 m*M* sodium octyl sulfate and 0.5 m*M* EDTA, to which 15% (v/v) methanol was added. The mobile phase was filtered through a

0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and then degassed under vacuum before use. A flow-rate of 1.2 ml/min (2500 p.s.i.) at ambient temperature was employed.

Preparation of standard solutions

Standard solutions of NE, E, DA, 5-HT, 5-HIAA, DHBA, and morphine (each 0.1 mg/ml expressed as the free base) were prepared in 0.1 *M* PCA, which had been previously filtered and degassed. These solutions were freshly prepared every two weeks; the morphine solution was stored at +4°C, and the other solutions at −70°C.

Animal procedures

Experiment I. Adult, male Sprague Dawley rats (Charles River, Montreal, Canada), weighing 250–290 g were housed individually in an environmentally controlled room at 21–23°C and 40% relative humidity, with lighting on from 7:00–19:00 h, for one week before use. They received food and water *ad libitum*, except that food was removed 24 h prior to sacrifice. On the day of experiment, the rats were injected intraperitoneally with 1 ml morphine (20 mg/kg) in saline or saline (per 100 g body weight). The rats were sacrificed by decapitation at 30, 75, and 120 min after the injection and the brains were removed rapidly. Each brain was dissected on an ice-chilled glass plate, and the striatum, hypothalamus, hippocampus, and midbrain-thalamus were separated, as described by Glowinski and Iversen[22]. The tissue was frozen on dry ice, and stored at −70°C until assayed. In order to determine whether the present technique is useful for determining morphine levels resulting from lower doses, rats were injected intraperitoneally with 5 mg/kg morphine and the midbrain-thalamus was dissected out and processed as described above. The cerebellum was saved to make a pooled cerebellum homogenate for use in the preparation of standard curves, as described below. An equal number of brains from each treatment (morphine or saline) and time group was processed each day in order to minimize variability of results.

Experiment II. Adult, male Sprague Dawley rats, weighing 245–260 g were kept under constant environmental conditions, as described above. Each rat was injected intraperitoneally with either 5, 10, or 20 mg of morphine (in 1.0 ml saline/100 g body weight). Blood samples (100 μl), collected from the tail vein at 15, 30, 60, 90, 120, and 180 min after injection, were carried through the entire procedure of Kim and Kats[24] for morphine determinations, using HPLC–ED.

Preparation of tissue samples for biogenic amines and morphine

Frozen tissue wet weights [mean ± standard error of the mean (S.E.M.)] were 65 ± 2, 69 ± 1, 107 ± 2, and 205 ± 5 mg for striatum, hypothalamus, hippocampus, and midbrain-thalamus, respectively. Individual samples were placed in polypropylene tubes, set in an ice-water bath, and were homogenized in 1.0 ml of 0.1 *M* PCA, containing 40 ng of DHBA as an internal standard, for 45 s by the use of a Polytron homogenizer (Brinkman Instrument, NY, U.S.A.). Homogenates were centrifuged at +4°C for 20 min at 35 550 *g*. The supernatants were separated and either immediately analyzed or stored at −70°C for assay later, within two weeks.

Supernatants were injected into the HPLC system, in a volume of 20 μl, by means of a 50-μl Hamilton syringe.

Standard curve

Known amounts (20–160 ng) of NE, E, DA, 5-HT, 5-HIAA and morphine in 1.0 ml aliquots of the pooled cerebellar homogenate (100 mg of tissue per ml of 0.1 *M* PCA) were taken through the entire procedure, DHBA (40 ng) being added to each of these samples as an internal standard. An identical set of samples of these compounds was made up in 1.0 ml of 0.1 *M* PCA. The measured levels of endogenous NE, E, DA, 5-HT, and 5-HIAA, contained in aliquots of the same homogenate without these added exogenous compounds, were subtracted from those of the corresponding standard samples. To construct the standard curve, the NE/DHBA, E/DHBA, DA/DHBA, 5-HT/DHBA, 5-HIAA/DHBA, and morphine/DHBA response ratios, corrected for the endogenous responses, were plotted against the amounts of each of these compounds, injected into the HPLC system. Actual amounts of these compounds, injected into the HPLC system, were 0.4–3.2 ng, while the amount of DHBA was 0.8 ng.

Statistical analysis

Data were analysed using one-way analysis of variance and Duncan's multiple range tests[23].

RESULTS AND DISCUSSION

Representative chromatograms of NE, E, DA, 5-HT, 5-HIAA, and morphine are shown in 0.1 *M* PCA (Fig. 1A) and in midbrain-thalamus homogenates, obtained from saline-treated (Fig. 1B) and morphine-treated (Fig. 1C) rats. No interfering endogenous compounds were apparent. Biogenic amines in frozen tissue samples were found to be completely stable when stored at −70°C for up to three months.

Limits of detection (*i.e.*, to give a recorder peak height of at least 0.1 a.u.f.s.) are summarized in Table I. The linearity of the concentration response relation for each of these compounds was established over the range of 20–160 ng in 1.0 ml of cerebellar homogenate (0.4–3.2 ng on column). Linear regression analysis from standard curves in cerebellar homogenates indicated that the correlation coefficients for all these compounds were higher than 0.9943. Recoveries of each of these compounds (20–160 ng) added to the homogenates were calculated by comparison of peak areas with results obtained with the corresponding samples in 0.1 *M* PCA, as well as with that of internal standard DHBA. The recoveries were essentially complete (Table I). The average between-run and within-run coefficients of variation for all these compounds were lower than 7.4 and 8.7%, respectively (Table I).

The ion-pairing reagent, 0.38 m*M* sodium octyl sulfate, permitted a large improvement in resolution. A recent report describes the principles and methodology of HPLC with ED and ion-pairing reagents[31]. We have found that a lower concentration, *i.e.* 0.14 m*M*, resulted in shortening the retention times for all compounds, but the resolution of 5-HT and morphine was poor. A higher (>20%) methanol concentration in the mobile phase also resulted in a poor resolution of both these compounds. In previous studies of HPLC methods for catecholamines and indoles[15],

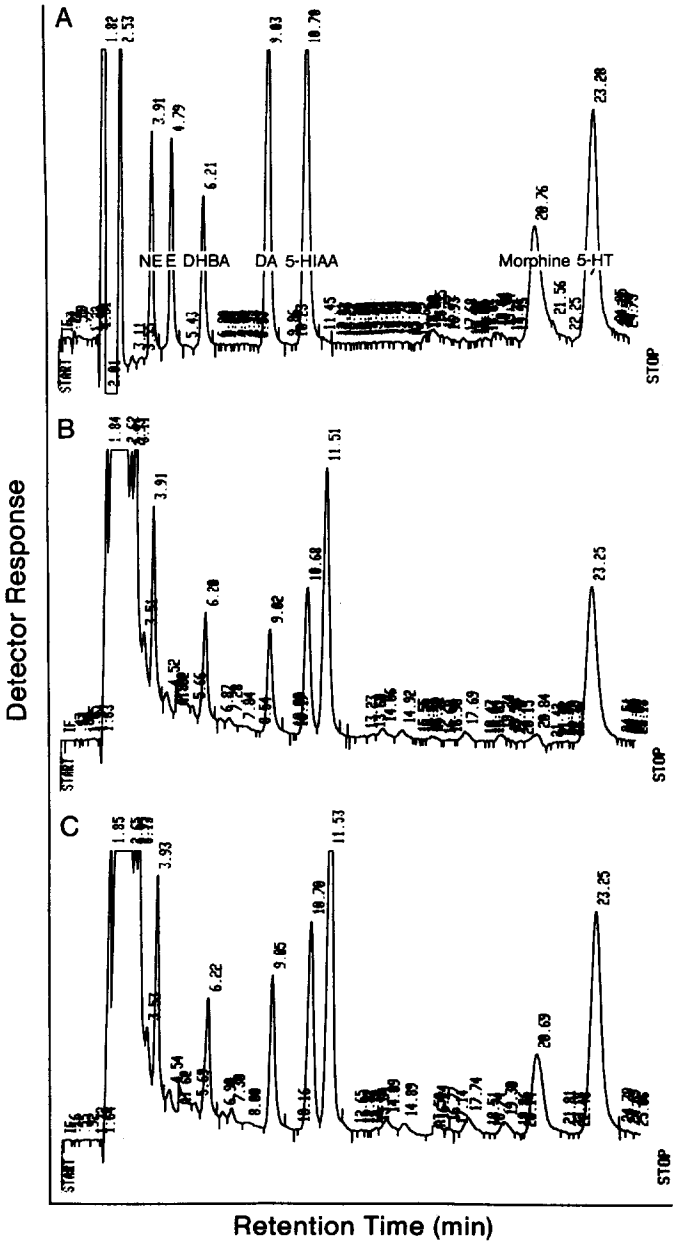

Fig. 1. (A) Reversed-phase HPLC separation of NE, E, DA, 5-HT, 5-HIAA and morphine standards (each 0.8 ng), and their internal standard DHBA (0.8 ng) in 0.1 *M* PCA). (B) Representative chromatogram demonstrating the HPLC separation and detection of NE, DA, 5-HT, and 5-HIAA from a portion of a single midbrain-thalamus obtained with a saline-treated rat. (C) Representative chromatogram demonstrating the HPLC separation and detection of NE, DA, 5-HT, 5-HIAA, and morphine from a portion of a single midbrain-thalamus obtained with a morphine-treated rat (20 mg/kg) at 30 min prior to sacrifice.

TABLE I

RECOVERY, PRECISION, AND LIMIT OF DETECTION OF THE HPLC DETERMINATION OF NE, E, DA, 5-HT, 5-HIAA AND MORPHINE IN CEREBELLAR HOMOGENATES

n = 5. C.V. = coefficient of variation.

Compound	*Recovery (%)*,** (mean ± S.D.)*	*Between-run C.V. (%)****	*Within-run C.V. (%)§*	*Detection limit (ng)*
NE	98 ± 7	5.6	7.7	0.1
E	94 ± 4	4.3	5.6	0.1
DA	100 ± 9	5.1	4.8	0.05
5-HT	110 ± 9	7.4	8.7	0.1
5-HIAA	106 ± 3	6.4	6.6	0.05
Morphine	108 ± 5	4.6	8.4	0.2

* Relative to the value for 0.1 M PCA solution.
** Over the range of 20–160 ng/ml homogenate.
*** Determinations over the range of 20–160 ng/ml homogenate.
§ Determinations at 20 ng/ml homogenate.

we had improved resolution at pH 4.2–4.5, which is a higher pH than is generally used. In the present work, varying the pH value of the mobile phase within the range of pH 4.2–5.0 did not critically influence the retention times for all compounds, and produced no differences in detector responses.

Different oxidation potentials (0.72–0.85 V) were also employed in the present study to obtain effective resolution. At a potential of 0.72 V, the detector response for biogenic amines was not decreased, but that for morphine was significantly decreased, and broadening of the peak was observed. At potentials of 0.8–0.85 V, the detector response for biogenic amines remained the same, but higher resolution for morphine was obtained. Under the experimental and mobile phase conditions described here, all compounds were readily oxidized at 0.8 V with sufficiently high cificity of analysis to detect biogenic amines and morphine simultaneously. The retention and resolution of biogenic amines and morphine were constant over 1000 runs on the same column. Maintenance of column efficiency was accomplished by (a) washing the column with 800–1000 ml of methanol–water (2:3) after 100–120 analyses and (b) replacing the guard column after every 250 analyses.

The concentrations of morphine in the hypothalamus of rats, sacrificed at 30 and 75 min after intraperitoneal administration, were significantly higher than those found in the midbrain-thalamus and striatum ($p < 0.001$ and $p < 0.025$, respectively), and the concentration of morphine in the hippocampus at 75 min was significantly higher than that of midbrain-thalamus or striatum ($p < 0.025$ and $p < 0.05$, respectively) (Fig. 2). The lowest morphine concentration measured in brain after a 20 mg/kg dose was 150 ng/g, in the 120 min sample of midbrain-thalamus (Fig. 2). This corresponds to an absolute sample size of about 0.6 ng on the column. The concentration of morphine in the midbrain-thalamus of rats sacrificed at 30 min after intraperitoneal administration of 5 mg/kg morphine was found to be 88 ± 5 ng (mean ± S.E.M., n = 5). Since the lower limit of detection is about 0.2 ng, it should be possible to measure tissue morphine values after doses in the 5–10 mg/kg range, *i.e.* after low analgesic rather than cataleptic doses. The peak levels of morphine

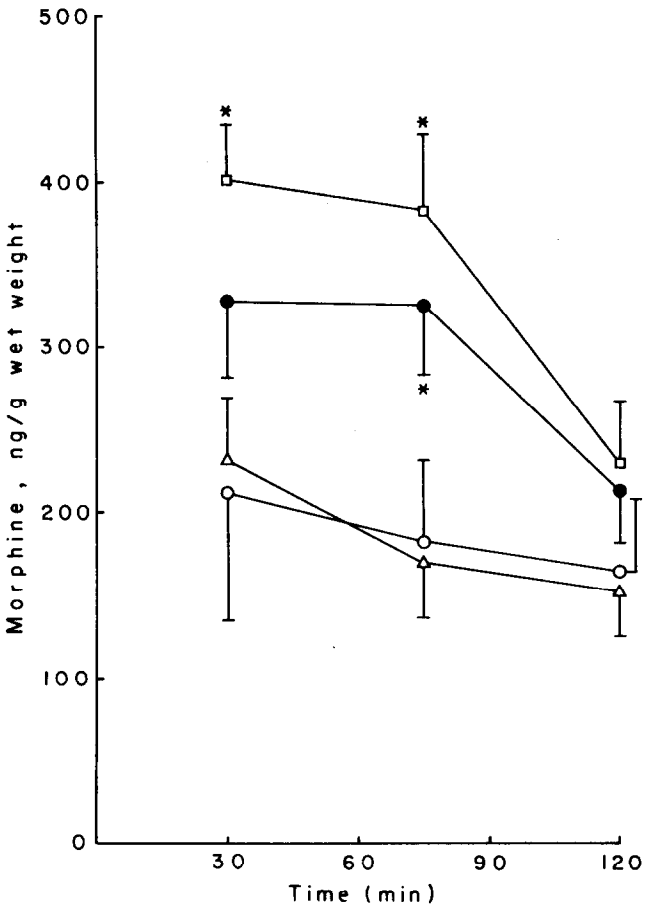

Fig. 2. Morphine concentrations in various brain regions of the rat at different time intervals after intraperitoneal injection of 20 mg/kg morphine: (□) hypothalamus, (●) hippocampus, (△) midbrain-thalamus, and (○) striatum. The points and vertical bars represent mean ± S.E.M. from four or five rats. An asterisk (*) means significantly different from midbrain-thalamus and striatum at p values 0.001, 0.025, or 0.05.

occurred at 30 min in all brain regions studied. The maximum blood level of morphine was also found to occur at 30 min after intraperitoneal administration of 5–20 mg/kg morphine (Fig. 3). Clearance of morphine from the blood appeared to follow an exponential time course, with an apparent biological half life of about 70 min, regardless of the dose given.

The baseline levels of biogenic amines found in the various brain regions in saline control rats are shown in Table II. The values found in hypothalamus, hippocampus, and midbrain-thalamus are in good agreement with those reported in the literature, but NE and DA levels in the striatum are 50–100% higher than most published values. The reason for the difference from other published values does not appear to lie in the HPLC technique, since it applies only to the striatum. Since our tissue weights for striatum were considerably lower than those reported by other

TABLE II

EFFECTS OF INTRAPERITONEAL MORPHINE ADMINISTRATION ON BIOGENIC AMINES IN VARIOUS BRAIN REGIONS OF THE RAT

A single intraperitoneal dose of either saline or morphine (20 mg/kg) was given at 30, 75, or 120 min prior to sacrifice. Since no statistical differences were found in the levels of biogenic amines between saline-treated rats at 30, 75, and 120 min prior to sacrifice, the data of the three groups were combined.

Experimental group	*Time of dose sacrifice (min)*	*Number of rats*	*Amount found (ng/g wet weight) (mean ± S.E.M.)*			
			NE	*DA*	*5-HT*	*5-HIAA*
Striatum						
Saline control		15	597 ± 39	15823 ± 514	728 ± 37	287 ± 15
Morphine	30	5	626 ± 61	16136 ± 1556	913 ± 108§	401 ± 70**
	75	5	697 ± 82	16720 ± 1200	728 ± 121	413 ± 63***
	120	5	599 ± 49	16667 ± 790	798 ± 46	383 ± 20§
Hypothalamus						
Saline control		15	3002 ± 123	462 ± 25	1023 ± 62	186 ± 19
Morphine	30	5	2487 ± 210*	413 ± 38	614 ± 51§	214 ± 18
	75	5	2396 ± 70***	465 ± 24	1016 ± 86	249 ± 28
	120	5	3134 ± 256	515 ± 36	950 ± 88	240 ± 35
Hippocampus						
Saline control		15	786 ± 36	43 ± 1	394 ± 22	103 ± 5
Morphine	30	5	814 ± 50	43 ± 4	422 ± 35	138 ± 12§
	75	5	897 ± 41*	46 ± 2	262 ± 11***	100 ± 11
	120	5	829 ± 38	45 ± 2	358 ± 26	151 ± 5§
Midbrain-thalamus						
Saline control		20	1184 ± 22	256 ± 8	1176 ± 33	210 ± 7
Morphine	30	10	1381 ± 54§	311 ± 12§	1385 ± 65**	284 ± 19§
	75	10	1181 ± 74	277 ± 26	1096 ± 32	236 ± 14
	120	10	1295 ± 33**	306 ± 10§	1423 ± 52§	341 ± 24§

* $p < 0.05$ compared to saline control.
** $p < 0.01$ compared to saline control.
*** $p < 0.025$ compared to saline control.
§ $p < 0.005$ compared to saline control.

investigators, it is conceivable that their larger samples contained substantial amounts of extra-striatal tissue with low catecholamine content, which diluted the NE and DA in the striatal tissue proper.

The effects of intraperitoneal administration of morphine on regional levels of biogenic amines are summarized in Table II. A significant increase in 5-HT at 30 min and a more sustained increase in 5-HIAA levels were found in the striatum, while NE and DA content in this region remained unchanged. In the hypothalamus, morphine administration resulted in a significant decrease in 5-HT and NE levels, with a return towards basal levels by 75–120 min. While NE and 5-HIAA levels were found to be increased in the hippocampus, a transient decrease in 5-HT concentration was noticeable 75 min after morphine administration. In the midbrain-thalamus both catecholaminergic and indoleaminergic transmissions were affected by morphine administration, *i.e.* NE, DA, 5-HT, and 5-HIAA levels were significantly increased at

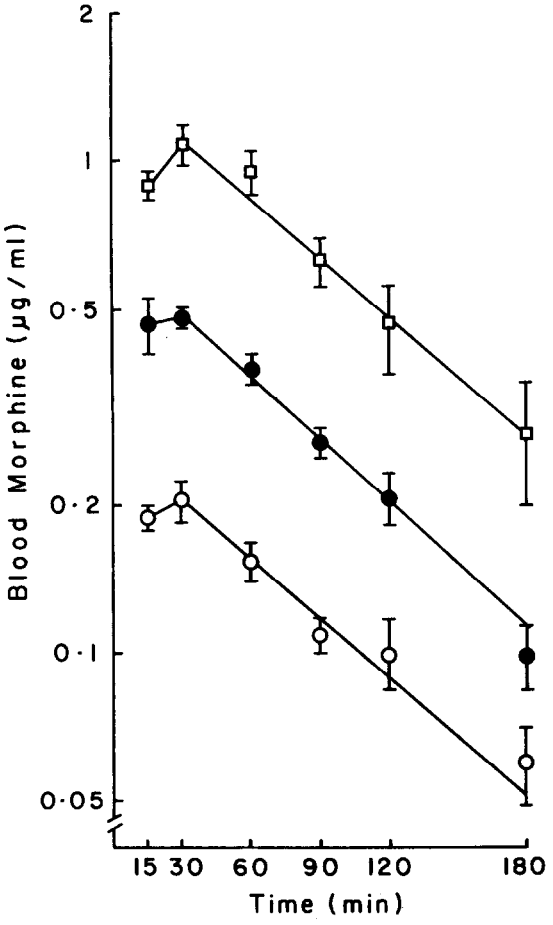

Fig. 3. Blood concentrations of morphine at various time intervals after intraperitoneal injection of 5 (○), 10 (●) or 20 (□) mg/kg of morphine. The points and vertical bars represent mean ± S.E.M. from five rats.

30 and 120 min, but there was a transient return to or below baseline levels at 75 min.

It is apparent that morphine influences the activities of catecholamines and indoles in different ways in various discrete brain regions. Our observation that acute morphine administration caused a decrease of NE level in the hypothalamus is consistent with findings by other investigators[25,26] and suggests that morphine acts either to stimulate NE release or to decrease its synthesis in this brain region. Johnson *et al.*[27] also reported that morphine administration produced slight, transient decreases in NE levels in most brain areas, and increased rates of NE biosynthesis in the hypothalamus. This combination of findings is more consistent with stimulation of NE release. On the other hand, other investigators have reported no changes in NE or DA levels in the hypothalamus[28] after acute morphine injection, or even a trend towards increased hypothalamic NE level[29]. However, we were unable to confirm a report of decreased NE level in the hippocampus[26]. Striatal DA was reported to be depleted and DA turnover rates increased in areas of striatum, hypothalamus, and midbrain[27]; in contrast, we found increased levels in the midbrain-thalamus, and no change in the other regions. Although steady-state concentration of 5-HT was not greatly affected by morphine administration, the level of 5-HIAA in the striatum was reported to be elevated[30]. Our findings are essentially in agreement with this report. The contradictory findings of morphine-induced enhancement or inhibition of biogenic amine release may indicate variability related to individual or strain differences, doses or morphine, routes of administration, or different environmental situations.

In the present study, the supernatant of the homogenates was injected directly into the column, eliminating the purification step of organic extraction. This eliminated a possible source of error, a loss in sensitivity and unsatisfactory recovery. The major advantages of the present method are simplicity of sample processing (elution time of 24 min for all these compounds), high sensitivity, and reproducibility sufficient to permit differential measurements of all of the substances in question in tiny samples from different regions of a single rat brain.

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