Biochemical Pharmacology, Vol 17, pp. 1627–1635. Pergamon Press. 1968. Printed in Great Britain

AN ULTRASENSITIVE METHOD FOR THE DETERMINATION OF MORPHINE AND ITS APPLICATION IN EXPERIMENTS *IN VITRO* AND *IN VIVO**

A. E. TAKEMORI

Department of Pharmacology, University of Minnesota, College of Medical Sciences, Minneapolis, Minn., U.S.A.

(*Received* 22 *January* 1968; *accepted* 1 *March* 1968)

Abstract—A very sensitive method has been developed to measure nanogram (ng) quantities of morphine in biological fluids. The morphine is extracted from biological fluids and the extract is completely dried in a vacuum evaporator. The extracted morphine is oxidized to pseudomorphine under mildly alkaline conditions. The fluorescence intensity of pseudomorphine is then determined in a filter fluorometer. The method has been used over a wide range of concentrations, and amounts as little as 10 ng can be accurately determined. This method has been used to determine plasma and cerebrospinal fluid levels of morphine in dogs after small i.v. doses of morphine sulfate. It has also been applied in the study of morphine transport by chloroid plexus *in vitro*.

METHODS for the estimation of morphine in biological fluids have been thoroughly reviewed by Way and Adler.[1] All of the photometric methods to date are only sensitive enough to estimate microgram amounts of morphine. Recently Kupferberg *et al.*[2] have reported a fluorometric method for the assay of morphine with which one could detect down to 0·1 μg. Radioactive methods[3,4] are even more sensitive, but are impractical in terms of cost and time for routine assays.

Previously, we had modified the fluorometric method of Kupferberg *et al.*[2] slightly so that a simple filter fluorometer could be used instead of a spectrofluorometer.[5] However, the modified method did not improve the sensitivity of the original method. The method described herein is a major modification of the fluorometric method and the method has been made at least ten times more sensitive than the original method. The main modification involved greatly decreasing the reaction volume and altering the conditions for oxidizing morphine to the highly fluorescent product, pseudomorphine. The details for the extraction of morphine from biological fluids and tissues are given. The practical use of this method in experiments *in vivo* and *in vitro* are also presented.

MATERIALS AND METHODS

Chemicals. All the common laboratory chemicals employed were reagent grade and the majority of them were bought from J. T. Baker Chemical Co. Chloroform was obtained from Baker and Adamson, Allied Chemicals. *n*-Butanol was bought

* This investigation was supported by United States Public Health Service Grants NB 05171 and GM 15477.

from Merck & Co. Tris, ultrapure grade, was procured from Mann Research Laboratories. All reagents were prepared in glass-distilled water which had been successively passed through commercial tanks of charcoal and deionizer resin (Culligan water conditioning) before distillation. Morphine sulfate was used in all experiments and the amount employed was calculated as the free base.

Glassware. All glassware used in this method should be scrupulously cleaned, preferably in hot detergent solution followed by soaking in a hot acid bath consisting of concentrated H_2SO_4 and HNO_3 in a 4:1 ratio. All glassware used in the extraction procedure was siliconized with Siliclad (Clay-Adams). The 5-ml centrifuge tubes used to evaporate samples should *not* be siliconized. The latter tubes should be clean enough so that 40–50 μl of aqueous fluid will completely drain to the bottom. The cleanliness of these tubes will not only insure against "bumping" when the samples are being evaporated under vacuum, but will permit maximum recovery of the morphine from the evaporated samples.

Extraction of morphine. One ml of plasma and 3 ml of freshly prepared 7% trichloroacetic acid are placed in a 12-ml centrifuge tube and mixed on a Vortex mixer. After centrifugation, 3 ml of clear supernatant is transferred to a 35-ml reaction vessel and 0·2 ml of 4·5 N NaOH, about 125 mg $NaHCO_3$ and 2 ml 0·5 M glycine buffer, pH 9·0, are added in order. The contents are mixed on a Vortex mixer and 10 ml of 10% *n*-butanol in chloroform is added. The vessel is stoppered with a No. 6 Caplug (Protective Closures Co., Inc.) and shaken on an Eberbach shaker for 10 min. After centrifugation to separate the phases, the upper aqueous phase is carefully removed by aspiration. Nine ml of the organic phase is transferred to a 12-ml centrifuge tube and 1·2 ml of 0·01 N HCl is added. The tube is stoppered with a Caplug, shaken on the Eberbach shaker for 10 min and centrifuged. One ml of the acid layer is transferred to a 5-ml centrifuge tube with a female joint accommodating Standard Taper No. 9 stoppers. The tube is attached to a Buchler Evapo-Mix, which has been modified to accept the smaller centrifuge tubes, and the acid extract is evaporated to dryness at 40° under vacuum. Several precautions should be taken to avoid "bumping" and consequent loss of the extract. The tube should be cleaned as recommended above and the vacuum should be placed on the tube gradually. The vacuum can be regulated conveniently by attaching a stopcock with a needle valve to the evaporator. The initial temperature of the bath should be about 0° and should be raised to 40° in a span of about 10–15 min. The tubes should be gently rotated on a horizontal plane throughout the evaporation process.

Analytical method. Forty μl of 0·5 M Tris-HCl buffer, pH 8·5, is added to the evaporated samples from above and mixed on a Lab-Line micro-mixer. The buffer should wash down the wall of the tube above the 1-ml mark to assure complete recovery of the evaporated extract. Two μl of 0·4 mM potassium ferricyanide is now added and mixed on the micro-mixer. After 15 min, 3 ml of distilled water is added and the resulting solution is read in a fluorometer.

Use of the filter fluorometer. The Turner fluorometer, model 111, equipped with a far ultraviolet lamp (General Electric No. G4T4-1), was used in most of the studies. Kupferberg *et al.*[2] showed by using a spectrofluorometer that the emission spectrum for pseudomorphine had a single peak at 440 mμ when determined with the excitation wavelength at 250 mμ, and the excitation spectrum had three peaks at 250, 280 and 320 mμ when determined with the emission wavelength at 440 mμ. In view of these

results, the following set of filters was chosen. The primary filter was No. 7–54 (Corning) which, when used with the far u.v. lamp, will result in excitation of samples at 254 mμ. The secondary filter was 47B (Kodak-Wratten) used with a 10 per cent neutral density filter (Kodak-Wratten No. 96). The secondary filter is a narrow pass filter which peaks at 436 mμ. The sensitivity range selector was usually set at 10×. It is important to note that quartz tubes must be used to read the samples.

The Farrand fluorometer, model A-3, with a microammeter can also be used. However, this instrument must be specially fitted with a quartz pencil lamp (BLE Spectronic Corp.) before it can be used for the present morphine assay.* Convenient instrument settings for determining morphine in the 10–100 ng range are to place the aperture setting of the fluorometer at 2 and the sensitivity setting on the microammeter at 3.

RESULTS

Effect of reaction volume. In the method of Kupferberg *et al.*,[2] the analytical reaction was performed in 2·1 ml and that in our modified method[5] was performed in 3·2 ml. Although only 70 per cent of the morphine was converted to pseudomorphine, this conversion was reproducible. Since pseudomorphine is a dimer and its formation involves molecular interaction, it occurred to us that morphine could be converted to pseudomorphine more readily by decreasing the reaction volume and concentrating the drug molecules. Thus, the conversion of morphine to pseudomorphine was carried out in a volume of 120 μl and the reaction mixture was subsequently diluted with 3 ml

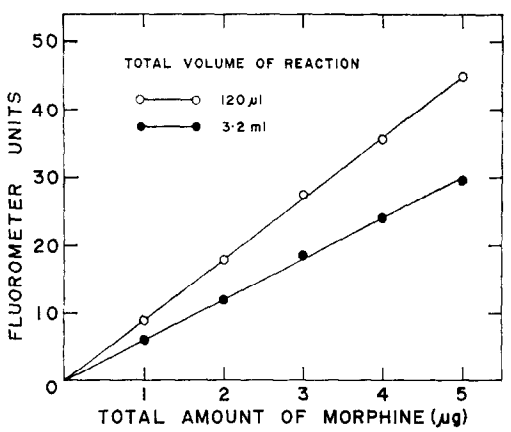

FIG. 1. Effect of reaction volume on the formation of pseudomorphine. The reaction performed in 3·2 ml contained 1 ml of an aqueous sample of morphine, 2 ml of 0·1 M Tris-HCl buffer, pH 8·5, and 0·2 ml of a solution consisting of 0·175 mM $K_3Fe(CN)_6$ and 0·0116 mM $K_4Fe(CN)_6$. The reaction performed in 120 μl contained 80 μl of an aqueous sample of morphine, 50 μl of 0·2 M Tris-HCl buffer, pH 8·5, and 20 μl of a solution consisting of 1·75 mM $K_3Fe(CN)_6$ and 0·119 mM $K_4Fe(CN)_6$.

distilled water and read on the fluorometer. Contents of the reaction mixture are described in the legend to Fig. 1. Tris-HCl buffer was used instead of the original pyrophosphate buffer,[2,5] since it was much more stable. The formation of pseudomorphine was markedly improved when the reaction was performed in 120 μl rather than in

* H. Kupferberg, personal communication.

3·2 ml. In fact, the amount of fluorescence produced in the larger volume was about 70 per cent of that produced in the smaller volume.

Although the standard curve for morphine appears linear when the formation of pseudomorphine is performed in 120 μl, the curve departed from linearity when the total amount to be estimated fell below 0·3 μg (Fig. 2). When the reaction volume was continually decreased, the standard curve again became linear when the reaction was performed in 42 μl. Since further decreases in reaction volume would have been impractical, it was decided to see how sensitive the method could be made by employing a reaction volume of 42 μl.

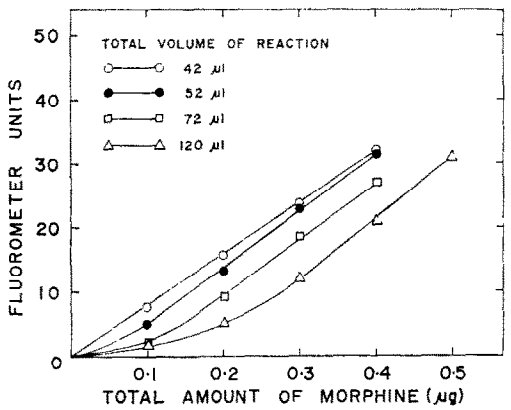

FIG. 2. Effect of reaction volume on the conversion of submicrogram amounts of morphine to pseudomorphine. The contents of the reaction mixture are those described for the reaction performed in 120 μl in Fig. 1. The amounts of the reagents employed were reduced proportionately as smaller total volumes were used.

Effect of ferricyanide and ferrocyanide on the formation of pseudomorphine. Kupferberg *et al.*[2] found that when a little ferrocyanide was added to the reaction mixture, the oxidation of morphine occurred more slowly and the product, pseudomorphine, was stabilized. They found that the optimal ferricyanide to ferrocyanide ratio to accomplish this was 16.

The effect of ferrocyanide on the formation and stability of pseudomorphine was investigated in more detail in the present study. With two concentrations of ferricyanide, the ratio of ferricyanide to ferrocyanide was varied from 5 to ∞. With 100 ng morphine in 40 μl of 0·5 M Tris-HCl buffer, pH 8·5, as the standard, the conversion of pseudomorphine was best accomplished in the absence of ferrocyanide (Table 1). Additionally, the fluorescence of the pseudomorphine formed in the absence of ferrocyanide was found to be stable for at least 1hr.

Since it was established that ferrocyanide was not required for the formation or stability of pseudomorphine, the effect of various amounts of ferricyanide on the oxidation of morphine was studied. The optimal amount of ferricyanide required to convert 10, 50 and 100 ng morphine, which represent $3{\cdot}5 \times 10^{-11}$, $1{\cdot}75 \times 10^{-10}$ and $3{\cdot}5 \times 10^{-10}$ mole respectively, was determined (Table 2). The conversion to pseudomorphine was not linear within this 10-fold range in the amount of morphine until 8×10^{-10} mole ferricyanide was used. Further increases in the amount of ferricyanide did not improve the conversion.

With the above conditions, a linear relationship between fluorescent intensity and the amount of morphine ranging from 10–100 ng was attainable (Fig. 3). This method is now approximately ten times more sensitive than the fluorometric method of Kupferberg *et al.*[2]

TABLE 1. FORMATION OF PSEUDOMORPHINE IN THE PRESENCE OF FERRICYANIDE AND FERROCYANIDE IN VARIOUS RATIOS*

$K_3Fe(CN)_6$	4×10^{-10} Mole $K_3Fe(CN)_6$		8×10^{-10} Mole $K_3Fe(CN)_6$	
$K_4Fe(CN)_6$	Moles of $K_4Fe(CN)_6$	Fluorometer* units†	Moles of $K_4Fe(CN)_6$	Fluorometer* unit†
5	8×10^{-11}	31·0	$1{\cdot}6 \times 10^{-10}$	32·0
10	4×10^{-11}	31·0	8×10^{-11}	37·5
20	2×10^{-11}	31·0	4×10^{-11}	40·0
40	1×10^{-11}	32·0	2×10^{-11}	42·0
∞	0	33·0	0	42·0

* The conversion of morphine to pseudomorphine was performed in 42 μl.
† Morphine, 100 ng, calculated as the free base was used as the standard.

TABLE 2. EFFECT OF VARIOUS AMOUNTS OF FERRICYANIDE ON THE CONVERSION OF MORPHINE TO PSEUDOMORPHINE*

Moles of $K_3Fe(CN)_6$	Morphine† (ng)	Fluorometer units
2×10^{-10}	10	3·5
	50	12·0
	100	18·5
4×10^{-10}	10	4·0
	50	15·5
	100	33·0
8×10^{-10}	10	4·5
	50	21·5
	100	42·5
$1{\cdot}2 \times 10^{-9}$	10	4·5
	50	20·5
	100	42·5

* The conversion of morphine to pseudomorphine was performed in 42 μl.
† Calculated as the free base.

Extraction of morphine. The final extraction procedure adopted for routine use has been presented under Materials ahd Methods. Many combinations of organic solvents were used to determine which solvent was best suited to extract morphine from the buffered, deproteinized plasma. The solvents that were used were 10% *n*-butanol, 10% ethanol or 10% *n*-amyl alcohol made in either chloroform or ethylene dichloride. Among the various combinations of solvents used for the extraction, 10% *n*-butanol in chloroform appeared to be the best.

The use of trichloroacetic acid to initially precipitate the plasma proteins and the siliconizing of the glassware were both very important in obtaining high, consistent

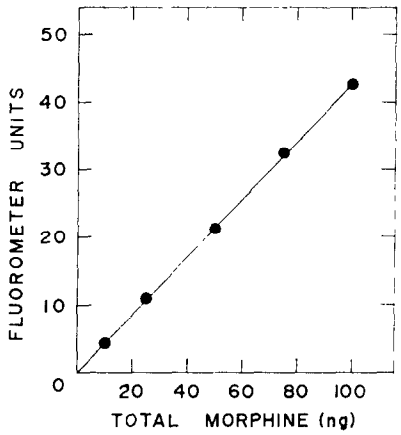

FIG. 3. Standard curve for morphine. The reaction mixture contained 20 μl ot the morphine sample 20 μl of 0·5 M Tris-HCl buffer, pH 8·5, and 2 μl of 0·4 mM $K_3Fe(CN)_6$.

recoveries of morphine (Table 3). Although good recoveries of morphine can be attained when the amount to be extracted is in the microgram range,[2,5] the recovery of morphine in the nanogram range was low and inconsistent when it was attempted without the use of trichloroacetic acid. The use of siliconized glassware improved the percentage of recovery by approximately 20 per cent.

TABLE 3. EFFECT OF TRICHLOROACETIC ACID AND SILICONIZED GLASSWARE ON THE RECOVERY OF MORPHINE FROM DOG PLASMA

Morphine (ng)* added to plasma	Per cent recovery ± S.E.†		
	Without trichloroacetic acid	With trichloroacetic acid	
		Nonsiliconized glassware	Siliconized glassware
25	29·2 ± 10·5	64·8 ± 5·8	86·6 ± 3·4
50	54·4 ± 6·1	62·2 ± 2·5	87·8 ± 3·3
100	46·1 ± 3·6	66·4 ± 2·5	87·0 ± 2·6

* Calculated as the free base.

† Per cent recovery = [Morphine (ng) recovered/morphine (ng) added to plasma] × 100.
The recoveries represent mean ± S.E. of determinations on 5–6 different plasma samples. Similar recoveries were observed with rat and human plasma.

The vacuum evaporation procedure of the final acid extract did not result in any loss of the narcotic. When 10, 25, 50, 75 and 100 ng morphine were added to 1-ml portions of 0·01 N HCl and evaporated, complete recoveries of the added morphine were obtained at all levels. However, the precautions mentioned under Methods must be heeded.

There are three steps in the complete extraction procedure where the original amount of morphine in the plasma sample is decreased by aliquot losses. Due to these losses, the amount of morphine in the final acid extract will represent 56·2 per cent of the

original amount in the 1-ml sample of plasma, and the estimated amount must be multiplied by a factor of 1·78.

Extraction of morphine from cerebrospinal fluid can be performed without the initial treatment with trichloracetic acid. The recoveries of added morphine from cerebrospinal fluid of dogs were very similar to those from plasma. In four experiments, the recoveries of 25, 50 and 100 ng added to cerebrospinal fluid were 89·0 ± 3·3 (S.E.), 90·2 ± 3·4 and 90·0 ± 2·9 per cent respectively. Additionally, since aliquot losses occur only in two steps of the extraction, the amount of morphine in the final acid extract will represent 75 per cent of the original amount in the 1-ml sample of cerebrospinal fluid.

The blank readings of the extract from plasma and cerebrospinal fluid were the same. The readings were very low and were about 50 per cent above the readings of distilled water.

Application in vitro; *Determination of morphine in choroid plexus.* Takemori and Stenwick[5] showed that morphine was actively taken up by the choroid plexus of rabbits *in vitro*. The new method allowed the use of much smaller pieces of choroid plexus and thus a larger number of samples could be obtained from a single animal. The method was used to study the uptake process and its inhibition by an antagonist. Levallorphan was conveniently used instead of the usual narcotic antagonist, nalorphine, since the latter antagonist interferes with the fluorometric determination of morphine while the former antagonist does not.[2] The procedure for studying the uptake of morphine has been described earlier.[5] After 1 hr of incubation, pieces of choroid plexus were homogenized in saline solution and an aliquot of the homogenate was taken for the extraction of morphine. The results were plotted according to the method of Lineweaver and Burk[6] and are presented in Fig. 4.

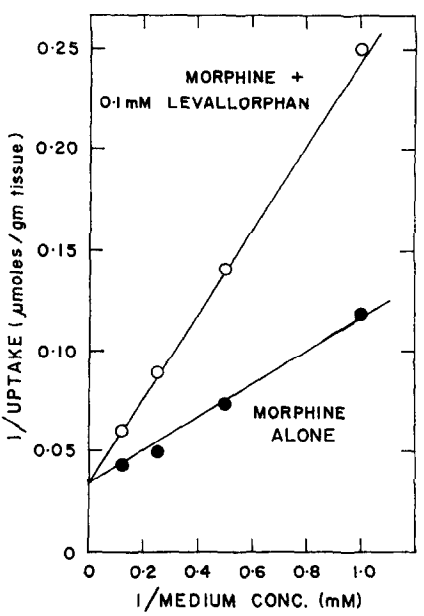

FIG. 4. The uptake of morphine by the choroid plexus of rabbits and the inhibition of this process by levallorphan. The pieces of choroid plexus were incubated for 1 hr at 37°.

The maximum rate of uptake of morphine by the choroid plexus was estimated to be 28·6 μmole/g tissue/hr, which corresponds very well with the value of about 25 μmole/g tissue/hr determined in an earlier study.[5] Levallorphan clearly showed a competitive type of inhibition of the uptake process as seen by the change of slope without a change in the maximum rate of uptake.

Application in vivo: *Determination of morphine in the plasma and cerebrospinal fluid.* A dog was anesthetized with an i.v. dose of 30 mg/kg sodium pentobarbital. The dog was intubated and artificially respired. A spinal needle was inserted into the cisterna magna for the collection of cerebrospinal fluid samples. After taking initial samples of cerebrospinal fluid and blood, the dog was given a dose of 0·5 mg/kg morphine sulfate i.v. Samples of cerebrospinal fluid and blood were taken at 5, 10 15 and 30 min after the injection of morphine. Enough blood was taken in heparinized syringes, usually 2·5 ml, to obtain 1 ml plasma. Morphine content of the plasma was determined as described under Methods. The 1-ml samples of cerebrospinal fluid were treated the same as the plasma, except that the addition of trichloroacetic acid in the extraction procedure was omitted.

Morphine appeared in the cerebrospinal fluid very rapidly, and as the plasma level fell the cerebrospinal fluid level continued to rise (Fig. 5). Very low levels of morphine in both the plasma and cerebrospinal fluid were easily determined. The lowest level of morphine detected in the cerebrospinal fluid at 5 min after injection was 26 ng/ml and that in the plasma at 30 min after injection was 25 ng/ml.

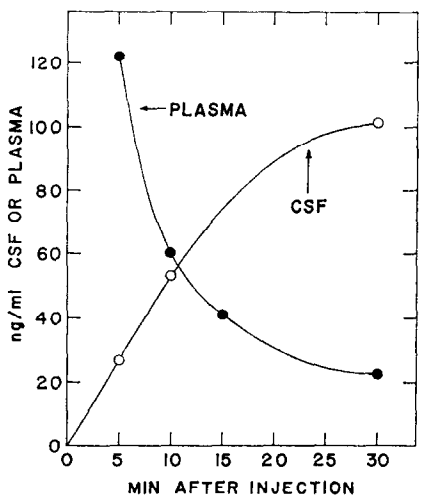

FIG. 5. The disappearance of morphine from plasma and the appearance of morphine in the cerebrospinal fluid (CSF).

DISCUSSION

The extraction of nanogram quantities of morphine from plasma presented several problems. Extraction of morphine without precipitating the plasma proteins resulted in poor recoveries of morphine. The use of trichloroacetic acid to initially precipitate the proteins made the recovery of morphine much higher and more consistent. This suggests that morphine is not bound very tightly to plasma proteins and much of the bound drug is released upon precipitation of the proteins.

Another problem encountered with working with such small quantities of the narcotic is the adsorption of the drug on the surface of the glassware. This problem can be circumvented somewhat by siliconizing the glassware. A substantial increase in the extraction of morphine occurred when siliconized glassware was used (Table 3).

Much of the increased sensitivity in the present method is attributed to the more complete conversion of morphine to pseudomorphine in very small reaction volumes. Data in Figs. 1 and 2 indicate that much more morphine was converted to pseudomorphine when the oxidation was performed in microliter rather than in milliliter volumes. The conversion was shown to be only 70 per cent complete when it was performed in milliliter volumes.[2] The fact that the fluorescence produced in 3·2 ml was approximately 70 per cent of that in 120 μl suggests that the conversion to pseudomorphine was complete in the smaller volume. It is also apparent that attempts to determine smaller amounts of morphine, i.e. nanogram amounts, require the use of even smaller reaction volumes to further concentrate the drug molecules.

The sensitivity of the fluorometric assay for morphine has been increased 10-fold and the method should be adequate for most determinations. It is possible, however, to increase the sensitivity further by selecting other neutral density filters or by using more sensitive fluorometers or by using both. The method described is, to our knowledge, the most sensitive nonradioactive method yet developed and has about the same sensitivity as radioactive tracer methods.[3,4]

Acknowledgements—I wish to thank Mrs. June Baumer and Mr. Loren Peterson for their excellent technical assistance and Dr. Harvey Kupgerberg for his interest and aid.

REFERENCES

1. E. L. Way and T. K. Adler, *Bull. Wld Hlth Org.* **25**, 227 (1961).
2. H. Kupferberg, A. Burkhalter and E. L. Way, *J. Pharmac. exp. Ther.* **145**, 247 (1964).
3. T. K. Adler, H. W. Elliott and R. George, *J. Pharmac. exp. Ther.* **120**, 475 (1957).
4. S. F. Mulé and L. A. Woods, *J. Pharmac. exp. Ther.* **136**, 232 (1962).
5. A. E. Takemori and M. W. Stenwick, *J. Pharmac. exp. Ther.* **154**, 586 (1966).
6. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56**, 658 (1934).