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Recovery of Morphine from Biological Samples by Hydrolysis and Solvent Extraction

Quantitative recovery of drugs from biological samples is important when a response is to be related to the amount of drug present in a tissue sample. Morphine is one drug of interest in this regard because of its widespread use and its chemical peculiarities. Its relatively low dosage, amphoteric nature, and metabolism to a water-soluble product, 3-morphine monoglucuronide [*1,2*], make morphine relatively difficult to analyze in biological samples. Most quantitative analytical schemes of analysis require that the glucuronide be cleaved to free morphine for extraction into an organic solvent. Acid hydrolysis [*3*] and enzymatic cleavage [*4*] are the most popular methods for freeing the morphine. We report here a study of the recoveries of radioactively tagged morphine from biological samples of morphine-treated dogs by using hydrolysis and solvent extraction. It is possible to recover more than 90% of the morphine contained in a sample with acid hydrolysis and about 80% with enzymatic cleavage.

Experimental Procedure

Morphine (*N*-methyl-^{14}C)·HCl was obtained from Mallinckrodt Chemical Works, and β-glucuronidase/aryl sulfatase, B grade, was obtained from Calbiochem. All other chemicals used were reagent grade.

Bray's solution consisting of 4 g PPO (2,5-diphenyloxazole), 0.2 g POPOP (2,2-*p*-phenylenebis-5-phenyloxazole), 60 g naphthalene, 100 ml methanol, 20 ml ethylene glycol, and 1,4-dioxane to a total volume of one litre [*5*] was used as the liquid scintillation solution.

Two systems of protein precipitants were used: (1) 5% w/v trichloroacetic and (2) 25% w/v sodium tungstate and 40% w/v sodium bisulfate added in equal volumes.

A nuclear Chicago Mark I liquid scintillation computer was used for counting ^{14}C in the channels ratio mode. Gas chromatography was done on a Finnigan Model 3000 gas chromatographic peak identifier. Eastman 6061 silica gel thin-layer chromatography plates without fluorescent indicator were developed in an Eastman 6071 developing chamber. A Waring blender and Thomas tissue grinding tubes with Teflon® plungers were used to homogenize tissue samples. An Exacta-Heat constant temperature block made by Technilab Instruments was used for enzymatic studies, and a Castle portable autoclave was used for hydrolyzing samples. A Fisher Roto-Rack was used to extract samples.

Because dogs have been shown to metabolize morphine similarly to humans [*1,2*], three dogs were injected intravenously or intramuscularly with an aqueous solution of morphine

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sulfate (20 to 50 mg/kg body weight) to which approximately 10 000 000 disintegrations per minute of morphine (*N*-methyl-^{14}C)·HCl had been added. After two or more hours had passed the animals were killed, and samples of blood, bile, urine, liver, and kidney were obtained. Blood was treated with potassium oxalate and sodium fluoride to prevent clotting and was immediately refrigerated with the other samples. From this pool, specimens were taken as necessary for analysis.

Results and Discussion

Solvent Systems

An evaluation of various organic solvent systems for extracting morphine from tissue samples was performed. One-millilitre portions of a single acid-hydrolyzed sample were made basic with ammonium hydroxide, saturated with sodium bicarbonate, and extracted with 10 ml of organic solvent. The results are shown in Table 1. Except where noted, this method was used to obtain the results reported herein. Based on the results obtained, the system of chloroform/isopropyl alcohol in the ratio of 4:1 was considered best because of its efficiency and its relatively high volatility, which is considered important for further analysis with concentration of the extract by evaporation, and was the solvent we used.

Sample-to-Solvent Ratio

Studies were also conducted to determine the relative efficiency of extraction of various ratios of sample to organic solvent. One-millilitre portions of an acid-hydrolyzed sample were extracted twice with 0.5, 1, 2, 3, and 4 ml of solvent as described above. The results are shown in Table 2. Extraction efficiency and precision were improved by increasing the amount of solvent. A solvent/sample ratio of at least three is recommended.

Effect of Heating and Hydrochloric Acid Concentration

The effects of heating samples to which various amounts of concentrated hydrochloric acid had been added were investigated. The effects of temperature and the amount of concentrated hydrochloric acid added to the sample were found to be interdependent. The results of these experiments are shown in Figs. 1 to 3. Equivalent yields of total morphine can be obtained with different techniques. Hydrolysis times of 45 min or less and overnight (15 h) were used. Samples were heated on a steam cone at 75 °C or in an autoclave at 103 kPa (15 psi) and 122 °C, allowed to cool to room temperature, made basic with ammonium hydroxide, saturated with sodium bicarbonate, and extracted twice with 10 ml of organic solvent. More acid must be added to a sample hydrolyzed at lower temperature to obtain equivalent recovery in the same time.

The amount of free drug present in a sample before treatment was determined to be from 6 to 12% of the total. To show that acid was necessary in addition to heat to cleave the glucuronide, controls with no added acid were run through the procedure. No change was noted in the value after the samples were heated with no acid added.

The results obtained with the autoclave experiment are in excellent agreement with those of Yeh and Woods [*6*], who obtained better than 90% recovery from similar samples.

Enzyme Hydrolysis

An alternate to the acid hydrolysis is to cleave the glucuronide with the enzyme β-glucuronidase. Curry [*4*] suggested that cleavage of the metabolites in this manner be carried out overnight at 37 °C with 4000 Fishman units of enzyme. The data plotted in Fig. 4 sug-

TABLE 1—*Relative*[a] *percentage of extraction efficiency of solvents.*

Mixture	Ratio	Trials							Average	Standard Deviation
		1	2	3	4	5	6	7		
$CH_2ClCH_2Cl/CH_3(CH_2)_3CH_2OH$	7:3	96	96	95	100	97	...	...	97	1.7
$CHCl_3/CH_3(CH_2)_3CH_2OH$	4:1	99	99	102	101	100	...	...	100	1.2
$CHCl_3/CH_3(CH_2)_2CH_2OH$	4:1	101	99	101	102	101	...	...	101	1.0
$C_6H_5CH_3/CH_3(CH_2)_2CH_2OH$	1:1	102	101	102	100	101	...	...	101	0.8
$CHCl_3/CH_3CH_2OCOCH_3/CH_3OH$	3:1:1	90	90	92	92	91	...	...	91	0.9
$CH_3CH_2OCOCH_3$	...	101	96	95	97	91	90	98	95	3.6
$CHCl_3$	...	98	87	93	93	90	83	88	90	4.5
$(CH_3CH_2)_2O$	...	78	69	69	73	69	66	70	71	3.6

[a]Relative to a mixture of $CHCL_3/CH_3CHOHCH_3$ in a 4:1 ratio, which yields 92% absolute recovery.

TABLE 2—*Relative[a] percentage of recovery of morphine by various ratios of solvent to sample.*

Ratio	Extractions	Trial 1	2	3	4	5	Average	Standard Deviation
0.5:1	first	56	73	78	77	64	70	8.4
0.5:1	second	34	22	17	16	26	23	6.6
1:1	first	65	70	77	86	86	77	8.4
1:1	second	31	28	21	11	12	21	8.1
2:1	first	85	88	92	90	89	89	2.3
2:1	second	16	15	12	9	11	13	2.6
3:1	first	92	95	95	93	95	94	1.3
3:1	second	10	9	4	5	5	7	2.4
4:1	first	97	98	97	96	100	98	1.4
4:1	second	3	3	3	4	3	3	0.4

[a]Relative to a single extraction of 1 ml of sample with 10 ml of solvent. Samples were saturated with sodium bicarbonate.

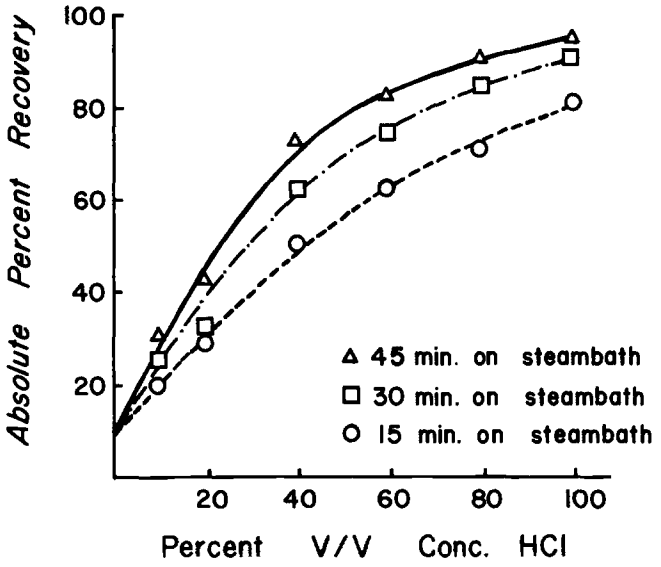

FIG. 1—*Recovery of morphine from acid-hydrolyzed urine and bile samples heated on a steam cone at 75°C for 15 to 45 min. Standard deviation of points is 4% or less.*

gest that it may take considerably more than 16 h to achieve results comparable to those obtained with acid hydrolysis. About 80% recovery can be obtained after the reaction has proceeded for 40 h. These results are somewhat higher than those obtained by Fish and Hays [7], who obtained about 60% recovery. This technique may be advantageous where destruction of other drugs to be analyzed may result with acid hydrolysis.

Salting-Out Agents

Various researchers [8,9] have used salts other than sodium bicarbonate for recovering drugs, including morphine, from biological samples by solvent extraction. They were con-

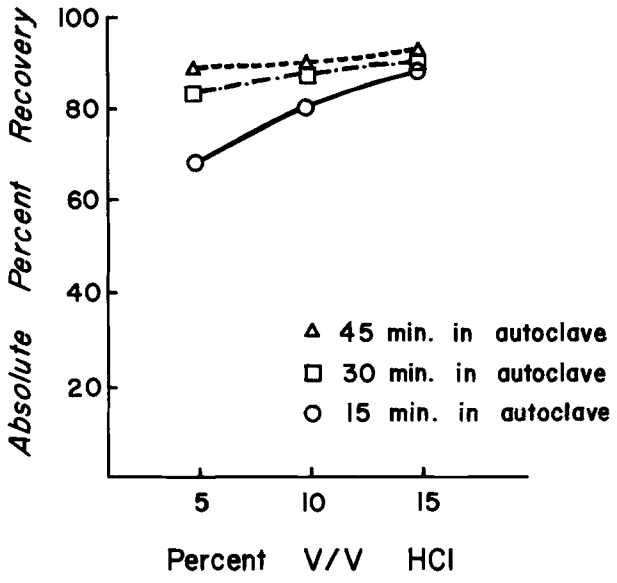

FIG. 2—*Recovery of morphine from acid-hydrolyzed urine and bile samples autoclaved at 103 kPa (15 psi) and 122°C for 15 to 45 min. Standard deviation of points is 3% or less.*

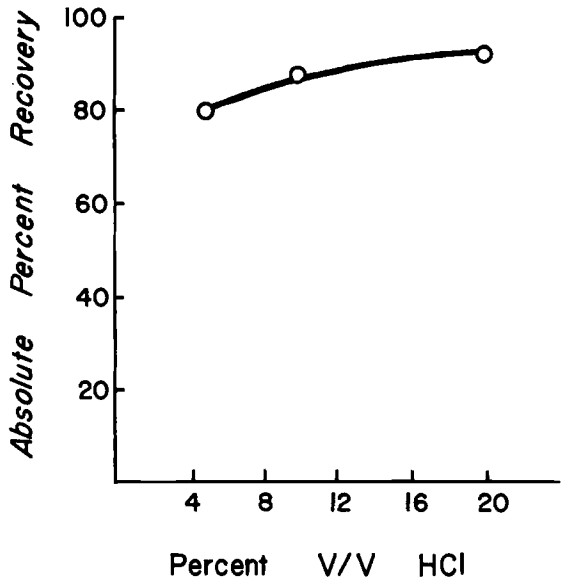

FIG. 3—*Recovery of morphine from acid-hydrolyzed urine and bile samples heated on a steam cone at 75°C overnight. Standard deviation of points is 3% or less.*

cerned with general screening procedures but found that morphine which had been added to urine samples could be recovered in good yield. Therefore, two other carbonates (ammonium and potassium) and sodium chloride were tested to ascertain effects they might have on recovery of morphine from urine and bile samples. In these experiments, aliquots

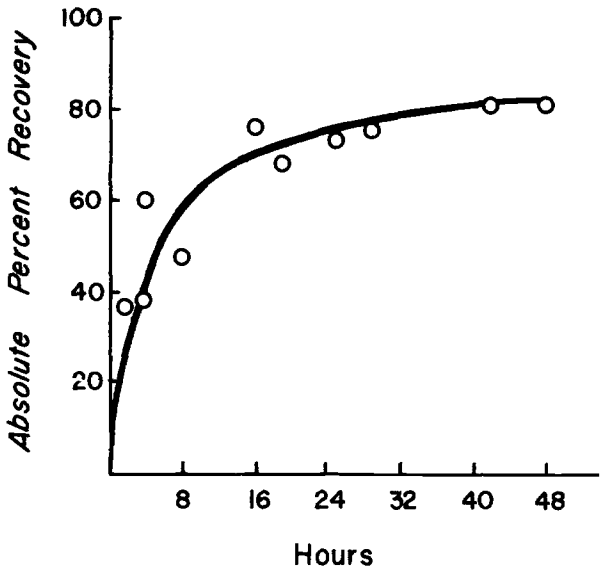

FIG. 4—*Recovery of morphine from samples treated with β-glucuronidase. Standard deviation of points is 6% or less.*

of a single acid-hydrolyzed sample were extracted in an identical manner except for the salt added. All samples were made basic with ammonium hydroxide, saturated with the salt of choice, and extracted with organic solvent. The results are shown in Table 3. The salting-out effect is universal for all salts used under the conditions of the experiment. Potassium carbonate was the only salt showing some variability in results. It is also the only one that forms a saturated solution more dense than the organic solvent. It becomes very viscous if more than a slight excess of salt is added, and this fact seems to account for the variability.

Protein Precipitation

Morphine was extracted from liver samples by three procedures, two of them using protein precipitation. Tungstic and trichloroacetic acids were the protein precipitants used. The third procedure was a 10% v/v hydrochloric acid digest on a steam cone overnight at 75°C. Autoclaving of tissue samples tends to result in boiled-over samples and therefore was not used in these experiments. The protein precipitation procedures yielded clear filtrates, and the acid digest procedure resulted in a brown solution that had a tendency to emulsify easily when extracted with organic solvents. The results are shown in Tables 4 and 5. Three-millilitre aliquots of a single hydrolyzed sample were extracted three times with 10 ml of organic solvent. Potassium carbonate was used as the salting-out agent. After protein precipitation, hydrolysis similar to that used on urine or bile samples was required because conditions for precipitation of proteins are not vigorous enough to cleave the glucuronide to any extent.

Blood samples were extracted in the same manner as liver samples. The results are shown in Table 6. The hydrochloric acid digest was assumed to yield 100% recovery since reliable scintillation counting could not be done on whole blood samples because of the low disintegrations per minute and excessive quenching. Therefore, the results shown can be considered only approximately correct.

TABLE 3—*Relative[a] percentage of extraction efficiency obtained with various salts.*

Trial	Salt		
	Ammonium Carbonate	Potassium Carbonate	Sodium Chloride
1	100	99	100
2	99	75	96
3	95	71	94
4	94	65 (99)[b]	100
5	98	(97)	97
6	98	(100)	...
Average	97	87 (97)	97
Standard deviation	2.1	12.9 (1.2)	2.3

[a]Relative to a sample saturated with sodium bicarbonate, which yields 92% absolute recovery.
[b]Percentages shown in parentheses were obtained when the solution was carefully saturated with only a slight excess of undissolved salt remaining in the extraction vial.

TABLE 4—*Percentage of recovery of morphine in the trichloroacetic (TCA) and tungstic acid (TA) filtrates of liver. These are compared to the hydrochloric acid digest given in Table 5.*

Filtrate[a]	Trial					Average	Standard Deviation
	1	2	3	4	5		
TCA_1	97	94	93	94	88	93	2.9
TCA_2	97	98	99	97	102	99	1.9
TA_1	54	51	50	53	56	53	2.1
TA_2	49	55	57	59	...	55	3.7

[a]Subscripts 1 and 2 represent heating for 15 h and 45 min, respectively.

TABLE 5—*Percentage of recovery of morphine from TCA and TA filtrates and from the hydrochloric acid digestion of liver.*

Trial	Extract[a]				
	Hydrochloric Acid	TCA_1	TCA_2	TA_1	TA_2
1	100	89	83	90	80
2	105	87	92	86	95
3	98	84	90	96	92
4	105	86	84	85	72
5	103	85	73	100	83
6	...	75	85	84	89
7	...	90	88	94	90
8	...	84	87	90	88
9	...	85	84	81	92
10	...	86	83	75	...
Average	102	85	87	88	87
Standard deviation	2.8	3.9	4.9	7.1	6.8
Total recovery[b]	102	79	86	47	48

[a]Subscripts 1 and 2 represent heating for 15 h and 45 min, respectively.
[b]Product of average values from Tables 4 and 5.

TABLE 6—*Recovery of morphine in TCA and TA filtrates* (a) *of blood, and recovery of morphine from extracts* (b) *of the filtrates. Recoveries in* (a) *and total recovery are compared to extracts of the hydrochloric acid digest.*

Method	Trial 1	2	3	4	5	Average	Standard Deviation	Total Recovery[a]
TCA a (filtrate)	75[b]	93	87	86	91	86	6.2	66
TCA b (extraction from filtrate)	80	74	78	75	80	77	2.5	
TA a (filtrate)	66	67	82	80	81	75	7.1	59
TA b (extraction of filtrate)	83	74	71	81	85	79	5.0	

[a]Product of average values of (a) and (b).
[b]Percentage recoveries.

Apomorphine Formation

Several authors report conversion of morphine to apomorphine [*6,7,10,11*] under some experimental conditions. No apomorphine was detected by either thin-layer chromatography or gas chromatography-mass spectroscopy with any of the procedures used in this paper.

Summary

Total morphine present in biological samples can be obtained by a variety of techniques. The method of Gross and Thompson [*3*] yields more than 90% recovery of the total morphine. The autoclave and steam cone (overnight) methods are preferred because of the small amount of mineral acid necessary to assure complete hydrolysis of the glucuronide.

Eighty percent recovery from urine can be obtained by using the enzyme β-glucuronidase for 40 h at 37°C. This method may be preferred when the conditions of acid hydrolysis might be deleterious to other drugs present in the sample.

Chloroform/isopropyl alcohol in the ratio of 4:1 seems to be the solvent of choice of those studied because it removes most of the morphine in a sample with a single extraction and it is readily volatile, which is considered important for concentration of the sample.

Several salts can be used to salt morphine out of aqueous ammoniacal solution. However, sodium bicarbonate and ammonium carbonate are preferred because they yield saturated solutions of a pH close to the isoelectric point of morphine, which is about 8.9 [*12*].

A ratio of 1 ml of sample to 4 ml of the organic solvent gives approximately 90% recovery of free morphine with a single extraction.

Quantitative results from tissues can be obtained by using the hydrochloric acid digest. However, the digest has a tendency to form emulsions unless the extraction is very gently performed.

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