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A CHROMATOGRAPHIC INVESTIGATION OF MORPHINE METABOLISM IN RATS

Confirmation of N-Demethylation of Morphine and Isolation of a New Metabolite

A. KLUTCH

New York State Department of Mental Hygiene

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ABSTRACT

Techniques based on thin-layer chromatography were applied qualitatively in an investigation into the metabolism of morphine in rats. The isolation of dihydromorphinone as a new metabolite of morphine is reported. The N-demethylation of morphine to normorphine was additionally confirmed during these investigations using several thin-layer chromatographic systems. The chromatographic method described was also applied to estimate the amounts of morphine and normorphine being excreted by rats treated with morphine.

The metabolism of morphine has been studied extensively in many species (1). It has been shown with the use of photometric methods that most of the dose of morphine given to man, dogs, and rats can be accounted for in the urine as unchanged morphine and its conjugates (2–5). More recently evidence of normorphine as a metabolic transformation product of morphine has also been reported (6, 7). Codeine has been isolated from the urine of morphine-treated rats (8).

In the course of studies on the metabolism of morphine, the present investigator developed a selective thin-layer chromatographic method for the analysis of morphine and the detection of biotransformation products in the urine of animals treated with the drug. In this communication we report the identification of dihydromorphinone as a new metabolite of morphine.

Materials and Methods

Chemicals. Morphine sulfate was obtained from Mallinckrodt Chemical Works (St. Louis Mo.) and codeine sulfate from Merck and Co., Inc. (Rahway, N. J.). Normorphine as the free base and norcodeine as the hydrochloride were supplied gratis for investigational use by Mr. Walter B. Gall of Merck, Sharp & Dohme Research Laboratories (Rahway, N. J.). Dihydromorphinone was a gift of Dr. Doris H. Clouet of the N.A.C.C. Testing and Research Laboratory (Brooklyn, N. Y.). All solvents used were of reagent grade standard as supplied by Fisher Scientific Co. (Pittsburgh, Pa.).

Animal Experiments. In a preliminary 8-day experiment, morphine sulfate dissolved in isotonic saline was administered intraperitoneally in single doses to four male Wistar rats (200 g) at a level of 10 mg/kg for 1 day and then at a level of 25 mg/kg for 7 days. Collections of urine were made for 24-hr periods during the administration of drug and were kept frozen before analysis. All urine for the above experiment was made up to 50 ml. Urine collected for the 24-hr period before drug administration was used as control urine.

In a large-scale metabolite-isolation experiment, which spanned 12 days, 12 male Wistar rats (200–225 g) were treated with successively increasing doses ranging from 25 mg/kg to 500 mg/kg. A total of 4.28 g was administered to the whole group of rats over the 12-day period.[1]

Urine was collected at 24-hr intervals during the entire period of the administration of drug. A 10% sample of each urine was removed and stored. The urine collected from days 4 through 12 was combined (1760 ml) and hydrolyzed as described below.

During the experiments, the rats were kept in stainless steel metabolism cages supplied by Acme Metal Products, Inc. (Chicago, Ill., Catalog Nos. AC 5162 and R_1-AC 5162). The baffle was modified to permit unre-

Send reprint requests to: Dr. Albert Klutch, Child Psychiatric Evaluation Research Unit, 524 Clarkson Ave., Brooklyn, N.Y. 11203.

[1] There was no loss of animals during experiment 1. However, during the large-scale experiment, three rats were lost due to fighting and cannibalism.

stricted flow of the urine, and the feces cup was replaced by a fine sliver of glass wool. The urine collection funnel was washed twice daily. The animals were fed Rockland Mouse and Rat Diet distributed by Teckland, Inc. (Monmouth, Ill.).

Determinations. *Urine Hydrolysis.* To 1.0 ml of rat urine, 1.0 ml of water and 0.2 ml of concentrated hydrochloric acid were added, and the solution was autoclaved for 90 min at 20 lb/in^2, a modification of the hydrolytic method described elsewhere (2). A modification of the original hydrolytic procedure has been reported previously (9). After the hydrolyzed urine was allowed to cool to room temperature, 0.32 ml of concentrated ammonium hydroxide and 8 ml of NH_3/NH_4Cl buffer, pH 9.5 (10), were added. The buffered urine (10 ml) was extracted with 50 ml of chloroform-isopropanol (4:1, v/v).

Initial Studies. For the detection of new metabolites of morphine during the initial qualitative experiments, 40 ml of the above organic solvent mixture were removed and evaporated under a stream of nitrogen while being heated gently in a water bath. The dried urine extracts were treated as described in the section on thin-layer chromatography. Control urine samples were treated similarly to the experimental samples in the preliminary studies. The reference standard used in these experiments consisted of morphine added to control urine in an appropriate concentration.

Detection of Normorphine. The detection of normorphine in the urine during the early qualitative experiments was best achieved by taking for analysis 20 ml of the mixed solvent extract described above. The quantitative estimation of normorphine in the experimental urine required from 1.0 ml to 10 ml of the solvent extract. The volume of sample taken from each extract depended on the concentration of the drug in each urine. These samples were estimated by comparison with reference standards which consisted of normorphine added to control urine in the appropriate concentrations and then carried through the hydrolytic procedure described. The dried urine extracts were treated as described below in the section on thin-layer chromatography.

Confirmation of Normorphine as Metabolite of Morphine. Experimental pools were obtained by combining an equal volume (1 ml) from each 24-hr urine sample of the above experiment. Urine from the experimental pool was hydrolyzed and extracted as described above. A urine sample from the experimental pool, to which 100 μg of normorphine had been added per ml, was treated similarly. To a third sample of the experimental pool hydrolyzed and extracted as described above, 100 μg each of codeine and norcodeine had been added per ml. A control urine sample containing 100 μg of normorphine per ml and a control sample containing 500 μg of morphine per ml were treated similarly. A 5-ml portion of solvent extract was taken from each sample of hydrolyzed urine and evaporated. The dried samples were treated as described in the section on thin-layer chromatography below.

Quantitative Estimation of Total and Unconjugated Morphine in Urine. The estimation of morphine was carried out on both hydrolyzed and unhydrolyzed urine. A 5-ml sample was withdrawn from each 24-hr urine of the first experiment and was diluted with 4 volumes (20 ml) of water. Two milliliters of this urine were hydrolyzed and buffered as described above. The buffered urine (10 ml) was extracted with 100 ml of chloroform.

The estimation of unconjugated morphine excreted by the animals given the lower doses of morphine in the first experiment required the undiluted urine. To 1 ml of the urine, 0.1 ml of 6 N ammonium hydroxide and 9 ml of the pH 9.5 ammonium hydroxide buffer were added. The buffered urine was extracted with 50 ml of the chloroform-isopropanol (4:1) mixture.

Control urine samples containing appropriate amounts of morphine were treated similarly. The standards in the control urine were in the same concentration range as that of the drug in the urine of drug-treated animals. From 1 to 10 ml of the solvent mixture were removed from each sample and evaporated. The dried samples were treated as described in the section on thin-layer chromatography. All urine analyses were done at least in quadruplicate and three chromatographic determinations were done on each analysis.

Thin-Layer Chromatography. Thin-layer plates were prepared from alumina GF supplied by Brinkmann Instruments Co. (Westbury, N.Y.). The techniques used in preparing the plates have been described previously (11).

A dried urine extract was dissolved in two drops of methanol added with a medicine dropper, and the total extract was applied to the thin-layer plate. The initial studies, including the detection of a new metabolite and normorphine, were carried out using system 1, described in table 1. Extracts to be examined quantitatively for morphine were chromatographed with system 1. Systems 1 and 2, table 1, were applied for the chromatographic estimation of normorphine, and the three chromatographic systems described in table 1 were also used for the identification of normorphine in the urine of the

TABLE 1

Solvent systems for thin-layer chromatography

Compound	R_F with System[a]		
	1	2	3
Normorphine	0.20	0.47	0.56
Morphine	0.40	0.80	0.90
Codeine	0.96	0.99	1.00
Norcodeine	0.49	0.57	0.64

[a] System 1, the lower phase of a chloroform/methanol/water/acetic acid (20:10:20:2) mixture; 2, the lower pmase of a chloroform/ethanol/water/acetic acid (20:10:15:1) mixture; 3, the lower phase of an ethylene dichloride/propanol/water/acetic acid (20:10:10:1) mixture.

animals treated with morphine. The extracts of the urine obtained from the drug-treated animals were chromatographed along with the hydrolyzed control urines and standards. The plates were dried at 80°C for 5 min, and cooled and visualized with the iodoplatinate reagent (12) diluted with an equal volume of 0.5 N hydrochloric acid. Maximum color development took approximately 15 min. However, the thin-layer plates being examined for the quantitative estimation of normorphine were not heated, but were allowed to cool in a fume hood at room temperature for 15 min before being sprayed with the iodoplatinate reagent. Maximum color development during the normorphine quantitation experiments took approximately 45 min. During the period of maximum color development, the drug concentration in the urine was estimated by a visual comparison with the standards. The best quantitative results were obtained when the urine sample applied contained from 1 μg to 2 μg of normorphine or from 0.5 μg to 1 μg of morphine.

Diazotized sulfanilic acid was employed as an aid in the identification of the isolated metabolic product. The reagent consisted of a solution of 2 g of sulfanilic acid and 5 ml of concentrated hydrochloric acid, diluted to 500 ml. Before use, 5 ml of the reagent were mixed with 1 ml of 1% sodium nitrite and allowed to stand for 5-10 min. The solution was made alkaline with 5 ml of 5% sodium carbonate. All reagents were kept in a refrigerator.

Isolation of Dihydromorphinone. A solution of 1760 ml of combined urine and 176 ml of concentrated hydrochloric acid was autoclaved at 20 lb/in^2 for 100 min. After the urine was allowed to cool to room temperature, sufficient concentrated NH_4OH (about 260 ml) was added to bring the pH to 9.5. The buffered urine was then extracted five times with 2.5 liters of a chloroform-isopropanol (4:1) mixture.[2] The aqueous phase was discarded, and the organic layers were combined and evaporated to dryness. The resulting residue was distributed in a mixture of 125 ml of NH_3/NH_4Cl buffer, pH 9.5, 1 ml of 6N NH_4OH, and 600 ml of chloroform, After the residue was shaken and the phases were allowed to separate, the chloroform phase was removed, and the aqueous solution was further extracted 5 times with 600 ml of chloroform.[2] After the aqueous phase was discarded, the six chloroform layers were combined and evaporated to dryness. The dried residue was treated as described below.

For a continuous serial extraction, four stoppered 50-ml capacity centrifuge tubes[3] were utilized. The dried residue described above was added to the first tube only. In addition, all four tubes received 20 ml of the lower phase of an ethylene dichloride/isopropanol/water/acetic acid (200:200:200:10) system. The extraction was accomplished by mixing each lower phase with 10 equal volumes of upper phase serially. The transfers were accomplished with a 25-ml volumetric pipet. When the extraction was completed, the lower phases were discarded and the upper phases were combined and evaporated. The residue was dissolved with a mixture of 60 ml of NH_3/NH_4Cl buffer, pH 9.5, and sufficient 6 N NH_4OH to neutralize any residual acetic acid (5-6 ml). The aqueous solution was now extracted six times with 300 ml of chloroform. The aqueous layer was discarded, and the organic solvent layers were combined and evaporated.

The residue was then purified in the following manner. Eleven centrifuge tubes, of the type described above, were arranged numerically from 0 to 10, each containing 20 ml of the lower phase of an ethylene dichloride/methanol/water (1:1:1) mixture. The residue which has been added to tube 0 was now subjected to a counter-current, single-withdrawal distribution with 16 equal volumes of the upper phase of the ethylene dichloride/methanol/water system being used to carry out the separation. The first five (lead) upper mobile phases (from transfers 11-15), which contained mostly morphine, were combined with the contents of tubes 9 and 10 and the upper phase of tube 8, and stored. The contents of tube 0 were discarded. The solvents from tubes 1 through 7 and the lower phase of tube 8 were combined and evaporated.[4] The above counter-current distribution was repeated on the residue. The contents of tubes 1 through 6 and the lower phase of tube 7 were combined and evaporated. The counter-current distribution was repeated a third and fourth time. Following the last distribution, the residue obtained after evaporation of the contents of tubes 1-6 and the lower phase of 7 was dissolved in methanol and filtered. After the methanol was removed by evaporation under a stream of nitrogen, the residue was treated with 2 ml of boiling water. The aqueous solution was then allowed to cool to room temperature, and the aqueous phase was filtered. This process was repeated twice on the above residue with 2 ml of water. The filtered aqueous phases were combined and evaporated. The dry residue was dissolved in 20 ml of hot toluene, and the organic phase was filtered. Next, the toluene was evaporated, and the residue was treated with 40 ml of boiling cyclohexane while it underwent considerable agitation. When the solid material was dissolved, the cyclohexane was concentrated to 5-10 ml and allowed to chill in a refrigerator (7-10°C). The cold solvent was then removed with a capillary pipet, and the residue that remained was dried very gently under a stream of nitrogen which yielded 16 mg of a white powder. The isolated metabolite was chromatographed using system 1, table 1.

The compound was analyzed by high-resolution mass spectrometry.[5] The ultraviolet spectra of the metabolite and dihydromorphinone—measured with a Beckman

[2] The pH of the aqueous phase was checked with a pH meter several times during the extraction.

[3] These tubes were obtained from Scientific Glass (Catalog No. JT-7950), Bloomfield, N. J.

[4] The progress of the separation was followed by examining minute amounts of each phase qualitatively by thin-layer chromatography using solvent system 1.

[5] The analysis by high-resolution mass spectrometry was done by Morgan Schaffer Corporation, 5110 Courtrai Ave., Montreal 252, Quebec, Canada.

model DK-2A recording spectrophotometer—had λ_{max} 280 nm and λ_{min} 262 nm in 0.1 N HCl and λ_{max} 292 nm and λ_{min} 274.5 nm in 0.2 N NaOH. The ultraviolet spectrum of morphine had λ_{max} 285 nm and λ_{min} 260 nm in 0.1 N HCl and λ_{max} 297 nm and λ_{min} 278 nm in 0.2 N NaOH. The infrared spectra of metabolite and reference dihydromorphinone—measured with a Perkin-Elmer model 237B infrared spectrophotometer—were identical. These spectra are shown in fig. 1. The infrared spectrum of morphine as the free base was also measured and is shown in fig. 2.[6] The infrared spectra were measured using KBr pellets.

Results

In the first experiment, four rats were treated with morphine for 8 days, and the urine that was collected was hydrolyzed with acid. During initial chromatographic studies on the extracts of the hydrolyzed urine, three substances were located (R_F = 0.20, 0.40, 0.75) when the developed thin-layer chromatogram was sprayed with the iodoplatinate reagent. The substance which yielded the most intense color with the spray reagent had a mobility (R_F 0.40) similar to that of morphine in the chromatographic solvent system being used (system 1, table 1). The results obtained for free and total morphine excreted daily by the rats are shown in table 2.[7]

A substance present in the urine, the mobility of which was slower than that of morphine in system 1, had R_F of 0.20 similar to that of normorphine. The previous observation (6, 7) that normorphine is present in the urine of morphine-treated rats was confirmed by chromatography. In each of three chromatographic systems, the following extracts were examined: a) a urine obtained from drug-treated animals or experimental urine, b) control urine, c) control urine with added morphine, d)

[6] Dihydromorphinone and morphine were purified using the extraction and distribution techniques described for the isolation of the new metabolite.

[7] In a second, 5-day experiment, 10 rats were given large doses of morphine which were increased on each successive day. Chromatographic investigation of the extracts from the urine excreted by the rats in this experiment indicated a greater percentage of the total morphine was excreted unconjugated in the second experiment, compared with the percentage of free morphine excreted in the previous experiment.

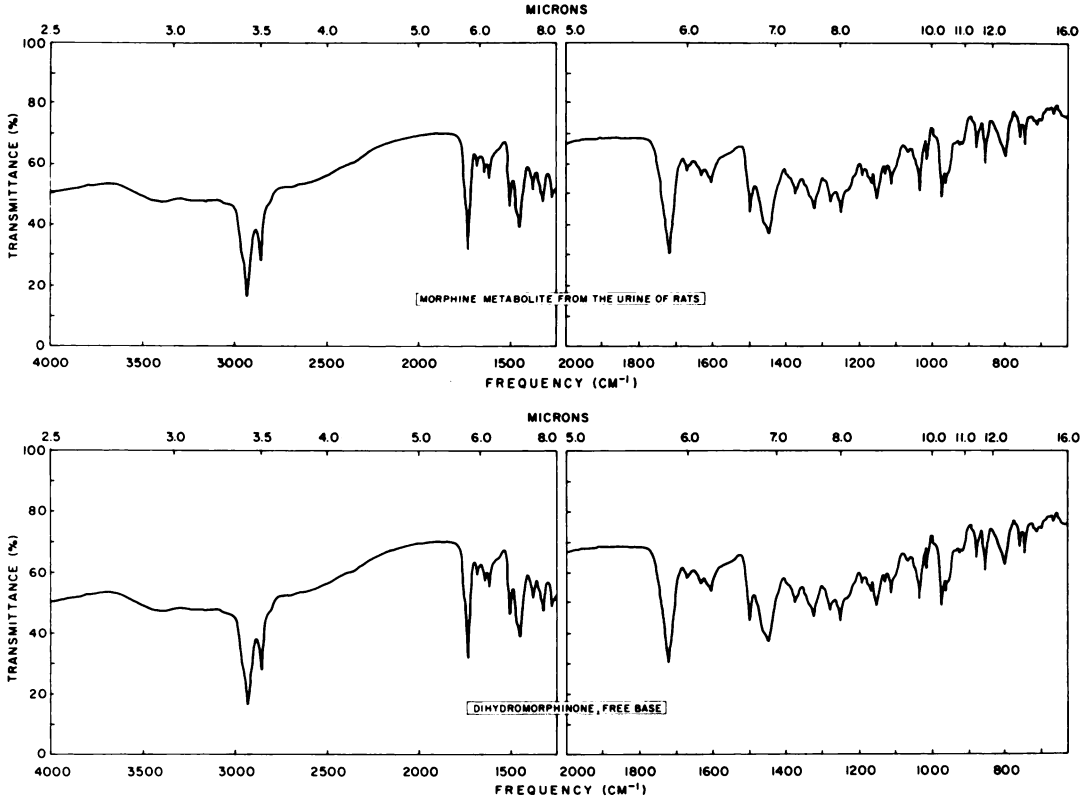

FIG. 1. *Infrared spectra of morphine metabolite and dihydromorphinone.*

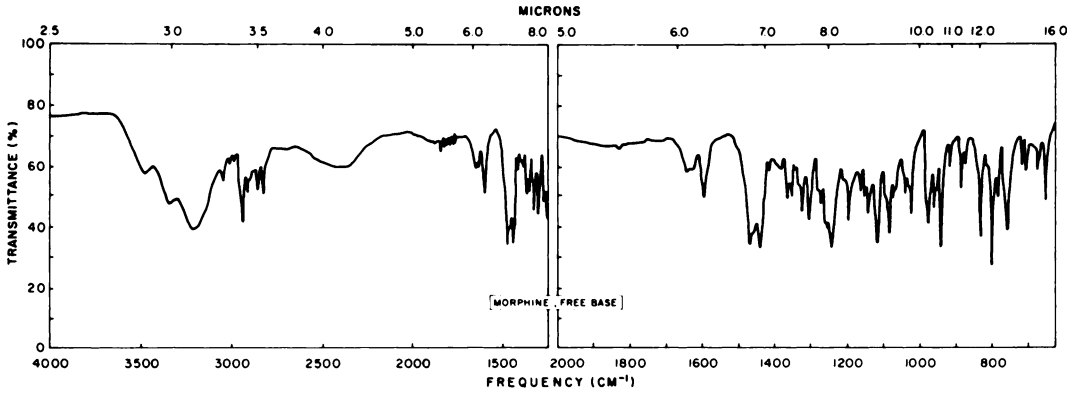

FIG. 2. *Infrared spectrum of morphine as the free base.*

TABLE 2

The estimation of morphine and normorphine in urine by thin-layer chromatography

Day	Free Morphine			Total Morphine			Normorphine		
	Mean[a]	Range[b]	Mean recovery	Mean[a]	Range[b]	Mean recovery	Mean[a]	Range[c]	Mean recovery
	μg/ml		*% of dose*	*μg/ml*		*% of dose*	*μg/ml*		*% of dose*
1	13 ± 2.0	10–15	8.1	70 ± 10.0	55–80	43.8	6 ± 2.0	4–9	3.8
2	29 ± 4.0	25–35	7.2	140 ± 14.0	120–160	35.0	15 ± 2.7	12–18	3.8
3	36 ± 4.0	30–40	9.0	210 ± 17.0	185–245	52.5	25 ± 3.4	20–30	6.3
4	39 ± 4.0	30–45	9.8	206 ± 15.0	180–240	51.5	22 ± 4.1	15–30	5.5
5	40 ± 4.0	35–45	10.1	195 ± 14.0	165–220	48.8	14 ± 3.3	10–20	3.5
6	40 ± 4.0	35–45	10.1	205 ± 16.0	190–240	51.2	12 ± 2.7	8–15	3.0
7	43 ± 5.0	35–45	10.7	230 ± 17.0	190–260	57.5	11 ± 2.3	8–15	2.8
8	45 ± 5.0	35–50	11.2	220 ± 17.0	190–260	55.0	10 ± 2.2	7–14	2.5

[a] Based on 12 determinations ± Student's SD of the mean.

[b] Sensitivity of method, 0.5 μg/ml.

[c] Sensitivity of method, 1.0–2.0 μg/ml.

control urine with added normorphine, e) experimental urine (obtained from the morphine-treated rats) with added normorphine,[8] and f) experimental urine with added norcodeine and codeine. Visualization by spraying a thin-layer plate demonstrated the presence of a substance with the same chromatographic mobility as normorphine in each of the experimental urine extracts and in the control urine to which it had been added. In the experimental urine to which norcodeine and codeine had been added, normorphine was well separated from norcodeine. There was no evidence for normorphine in the control urine and in the control urine to which morphine had been added. This last finding confirms the observation that normorphine is a metabolic transformation product of morphine, and indicates that its detection in the experimental urines was not caused by an artifact, such as chromatographic degradation of morphine. Codeine, which moves with the solvent front in all of the thin-layer solvent systems, was not detected in any of the experimental urines.[9] However, a substance with the same chromatographic mobility in system 1 as that of codeine was present in low concentration in the control morphine standards.

The total amount of normorphine excreted daily was also estimated with a thin-layer chromatographic method, and the results are shown in table 2. The values obtained by this method (2–6%) are in general agreement with values (6–7%) previously reported (6, 7).

It was observed in the above experiment that

[8] These samples may be described as internal standards which have been discussed previously (see ref. 11 and footnote 10).

[9] Despite the fact that codeine has been isolated from the urine of male, Long-Evans rats (8), codeine could not be detected in urine of the experimental animals used in this experiment.

there is present in the urine a substance with an R_F (0.75) greater than that of morphine in solvent system 1 (table 1). The mobility of this substance was different from that of normorphine, of codeine, and of norcodeine, which indicates the presence of a new metabolite of morphine. In order to explore the possibility that there was a new metabolite of morphine in the urines of our morphine-treated rats, a new experiment was carried out in which large doses of morphine were given to the animals. It was anticipated that the massive amounts of the alkaloid given to the animals would result in an increased excretion of this substance in quantities sufficient to be isolated and that its identity as a new metabolite of morphine would be consequently established. Urine collected from the animals was hydrolyzed with acid and extracted with a chloroform-isopropanol mixture. Although the alkaloid material is extracted quantitatively from a large volume of urine in this way, it is probably obtained as a salt.[10] The urine extract was redissolved in pH 9.5 buffer, and the morphine-related compounds were reextracted with chloroform. Although the drugs are not extracted as well in this way as with the more polar chloroform-isopropanol mixture, chloroform does extract morphine as a free base leaving behind inorganic salts and other polar compounds.

In a preliminary serial extraction with an ethylene dichloride/isopropanol/water/acetic acid system, the alkaloids were separated from the large bulk of the organic urine constituents. After the drugs were freed from their acetate salts, the metabolite was separated from morphine with manual counter-current distributions, using an ethylene dichloride/methanol/water system. A white, powdery substance was obtained by a purification procedure which involved filtration and a modified recrystallization procedure from cyclohexane. Thin-layer chromatography studies on the isolated material indicated that the substance was pure and free of contamination with morphine. The metabolite gave a stable, light orange color when sprayed with the diazotized sulfanilic acid reagent after having been chromatographed using system 1, table 1. The phenolic compounds, morphine and normorphine, gave a similar reaction with the spray reagent; codeine gave a transient yellow color, and norcodeine did not react with the diazotized sulfanilic acid. All compounds yielded dark blue colors after being sprayed with the iodoplatinate reagent.

TABLE 3

Comparison of the mass spectra of the new metabolite and morphine

The mass and the relative intensity of the major fragments found in the mass spectra are shown.

Metabolite		Morphine	
m/e	Relative intensity	*m/e*	Relative intensity
285	100	285	100
229	38.3	284	17.7
228	37.2	268	15.2
214	24.5	215	33.9
200	23.4	174	18.5
170	23.4		

A molecular weight of 285 for the compound was obtained by high-resolution mass spectrometry. The isotopic analysis was in good agreement with the theoretical value for the molecular formula $C_{17}H_{19}NO_3$.[11] The molecular formula and the chromatographic data indicated that the metabolite was an isomer of morphine. This conclusion is supported by the presence of a major peak at *m/e* 229 in the mass spectrum of the compound, whereas the second most intense peak in the mass spectrum of morphine is at *m/e* 215.[12] The mass of the major fragments found in the mass spectrum of the metabolite and morphine is shown in Table 3.

The bathochromic shift in the ultraviolet spectrum of the compound in going from acid to alkaline solution is a result expected from a compound containing a phenolic hydroxyl.

The infrared spectrum of the metabolite had a peak for a carbonyl group at 1780 cm^{-1}. The spectral data obtained on the compound suggested that the compound isolated was dihydromorphinone. The infrared spectrum of dihydromorphinone was measured, and it was found to be identical with the spectrum of the metabolite. When techniques similar to those described for the estimation of morphine and normorphine were used, preliminary studies indicated that dihydromorphinone accounted for approximately 4% of the dose given to rats in experiment 1.[13]

[10] When synthetic morphine was extracted with the chloroform/isopropanol mixture and distributed in the ethylene dichloride/methanol/water system described in Materials and Methods, the base was found concentrated in the lead, upper mobile phase 0.

[11] The results were interpreted by Dr. Robert A. Schaffer of the Morgan-Schaffer Corp.

[12] A copy of the mass spectrum of morphine published by the Shell Oil Co. was supplied through the courtesy of Dr. Robert A. Schaffer.

[13] Preliminary experiments indicate that the compound is present in the urine of addicted human subjects.

Discussion

In a recent publication the two types of metabolic changes undergone by drugs have been discussed (13). In this work we have been mainly concerned with the biotransformations of morphine in rats, inasmuch as the conjugation reactions of morphine, codeine, dihydromorphine, and normorphine have been studied extensively (2 5, 8, 14-16).

Chromatographic studies on the metabolism of morphine indicated the presence of a metabolite having a chromatographic mobility slower than that of the morphine. Among the possibilities considered for the identification for this metabolite was normorphine, because a wide variety of compounds have been demonstrated to undergo N-dealkylation in the intact animal as well as *in vitro* (17).

Indirect evidence for the N-demethylation of morphine *in vitro* has been provided from tracer studies (18). More direct approaches have been used to show the formation of normorphine from morphine *in vitro* (6, 7). These methods involved the identification of normorphine from its mobility on buffered paper chromatograms. However, for only one chromatographic system has there been reported a significant difference in the chromatographic mobilities of morphine and normorphine (6). In the present work, the three thin-layer chromatographic solvent systems described provided for bettern separation of morphine and normorphine and were employed for the confirmation of the N-demethylation of morphine.

Evidence for dihydromorphinone as a metabolite of morphine has also been presented by the present study. Visual examination of the thin-layer plates during the first experiment had indicated that the new compound was present in about the same concentration as normorphine. In a large-scale experiment, sufficient material was obtained to permit identification of the new metabolite.

The mass spectral data showed that the molecular weight of the compound and morphine was the same. However, the fragmentation patterns of the two compounds were quite different.[14]

Ultraviolet data on the new metabolite suggested that the substance isolated contained a phenolic group. However, the maximum and minimum wavelengths of the new metabolite were different from that of morphine. The reaction of the metabolite with the sulfanilic acid reagent was consistent with the presence of a free phenolic group. Because the ultraviolet data of the new metabolite resembled that published for dihydromorphinone (19), a sample of the compound was obtained. The ultraviolet and the infrared spectra of both compounds were measured and found to be identical.

The techniques and limitations of the quantitation of a drug and its metabolic transformation products in the urine of animals treated with the drug have been described elsewhere (20). Morphine and normorphine were conveniently estimated in many urine samples by the method described in Materials and Methods. Solvent systems 1 and 2 (table 1) were used for the quantitative work because development of a thin-layer plate with these systems resulted in the least interference of urine contaminants with the visualization of chromatographed drug. The use of the chromatographic systems described above imparts great specificity and sensitivity to the present method. Nevertheless, results obtained by our method are not as precise as those obtained by the earlier methods (2, 3, 6).

The variability in the results shown in table 2 arises mainly from the last step in the analysis, because it is difficult to apply urine extracts uniformly to thin-layer plates. The application of gas chromatography in the last stage of the analysis would probably sacrifice sensitivity, but it would render the method more precise. However, the method as now constituted can be used in many routine investigations inasmuch as it provides a reliable, accurate estimation of the unmetabolized drug and of the relative amounts of normorphine and dihydromorphinone present in the urine of animals being treated with the drug.

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[14] Indeed, the mass spectra of morphine, normorphine, codeine, and norcodeine show striking similarities. The mass spectrum of the new metabolite was very different from any of the above compounds.

Nutley, N. J.) for the mass spectrum of an early impure sample of the metabolite.

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