Separation and Identification of Morphine and Its Metabolites and Congeners

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Abstract □ Morphine and its known and postulated metabolites and congeners, *i.e.*, morphine *N*-oxide, normorphine, pseudomorphine, morphine-*N*-methyl iodide, codeine, norcodeine, morphine-3-glucuronide, and morphine ethereal sulfate, were separated by TLC and GLC. The R_f values of nalorphine and nicotine and its metabolites and the retention times of nalorphine and seven other chemicals commonly used for GLC quantitative determination of morphine and codeine are also presented.

Keyphrases □ Morphine and metabolites—separation, identification, TLC and GLC □ TLC—separation, identification, morphine and metabolites □ GLC—separation, identification, morphine and metabolites

Studies in various animal species have shown that morphine is biotransformed to morphine-3- and 6-glucuronide (1–5), morphine-3-ethereal sulfate (6–8), morphine *N*-oxide (9, 10), normorphine (11–14), and codeine (15). Pseudomorphine (16), an oxidation product of morphine, and morphine glutathione (17) have also been postulated to be metabolites.

While investigating the pharmacokinetics and metabolism of morphine in man, need arose for the development of suitable methods for separation and identification of morphine and its metabolites. Although many procedures, including TLC and GLC, have been described for separation of morphine from its congeners, as mentioned in recent reviews (18, 19), none has been described for separation of morphine from its metabolites. This report describes the application of TLC and GLC for the separation and identification of these compounds.

EXPERIMENTAL

Morphine sulfate USP[1], codeine phosphate USP[1], and normorphine hydrochloride[2] were obtained. Pseudomorphine, morphine *N*-oxide, and morphine-*N*-methyl iodide were prepared by previously described methods (20–22). Morphine-3-glucuronide and morphine-3-ethereal sulfate were isolated from the urine of dog and cat, respectively (4, 8).

TLC[3]—The compounds were localized after development by spraying with potassium iodoplatinate reagent or ninhydrin reagent.

GLC—A chromatograph[4] equipped with dual-flame-ionization detectors and dual-pen recorders[5] was used for GLC. The columns (Table II) were conditioned at 275° under nitrogen, 30 ml./min., for 1 hr.; then at 340° without nitrogen for 4 hr.; and finally at 290° with nitrogen, 16 ml./min., for 72 hr. The temperatures of injector and detector were set at 255 and 295°, respectively; gas flow rates were 30 ml./min. for nitrogen and hydrogen and 300–400 ml./min. for compressed dried air.

Silylation of Morphine and Its Metabolites and Congeners—Methanol solutions of morphine, codeine, normorphine, norcodeine, morphine *N*-oxide, and morphine-*N*-methyl iodide were placed in acylation tubes[6] and evaporated to dryness under nitrogen in a water bath at 60–70°. To the residues were added 0.05 ml. acetonitrile and 0.05 ml. of bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane[7]. The tubes were capped and shaken on a Vortex mixer for about 10 sec. After heating at 60–70° for 30 min., 1 μl. of the mixture was injected into the gas chromatograph.

Acetylation of Morphine and Its Metabolites and Congeners—Methanol solutions of morphine, codeine, normorphine, norcodeine, morphine *N*-oxide, and morphine-*N*-methyl iodide were placed in acylation tubes and evaporated to dryness under nitrogen in a water bath at 60–70°. The residues were either trifluoroacetylated with 0.2 ml. of trifluoroacetic anhydride or acetylated with 0.2 ml. of acetic anhydride and 0.1 ml. of pyridine. The tubes were sealed with screw caps, and the mixtures were warmed for 30 min. in an oil bath at 60–70°. Excess trifluoroacetic anhydride or acetic anhydride was removed under a stream of nitrogen in an oil bath. The residues were dissolved with 0.1 ml. of ethyl acetate by shaking on a Vortex mixer for 10 sec. One microliter of the solutions was injected into the gas chromatograph.

RESULTS AND DISCUSSION

TLC Separation of Morphine and Its Metabolites and Congeners—The R_f values of morphine and its metabolites, obtained with various thin-layer plates and developing solvent systems, are presented in Table I. A good separation of morphine from other free compounds, with the exception of norcodeine, was obtained with the silica gel precoated plates developed with a solvent mixture of ethyl acetate–methanol–ammonium hydroxide (17:2:1) or with the instant silica gel sheet developed with a mixture of *n*-butanol–*n*-

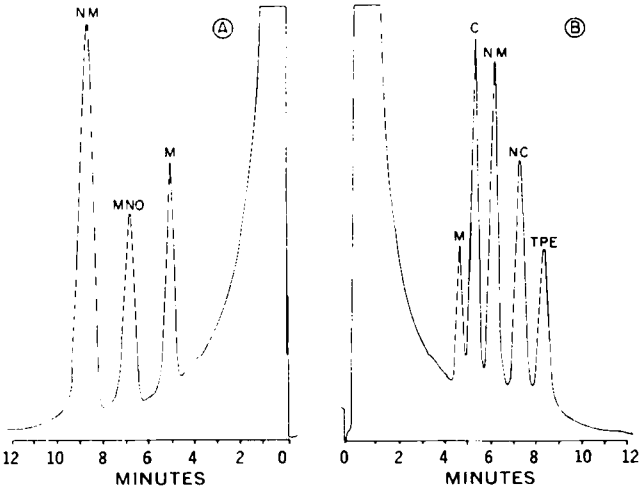

Figure 1—*Gas chromatograms of trifluoroacetyl derivative of morphine (M), codeine (C), normorphine (NM), norcodeine (NC), morphine N-oxide (MNO), and tetraphenylethylene (TPE). Key: A, obtained from Column 2; and B, obtained from Column 1 at 205°.*

[1] Merck Sharp & Dohme.
[2] Through the courtesy of Dr. Everett May, National Institutes of Health.
[3] Gelman instant thin-layer silica gel sheets (ITLC-SG type, Gelman Instrument Co., Ann Arbor, Mich.), and Quantum silica gel precoated plates were used for TLC, with application of standard techniques.
[4] Varian Aerograph, Series 2700.
[5] Model A-25.
[6] Regis Chemical Co.
[7] Regisil TMCS (10%), Regis Chemical Co., Chicago, Ill.

Table I—R_f (× 100) Values of Morphine and Its Metabolites

Substances	Instant Thin-Layer Silica Gel Sheet: Ethyl Acetate–Methanol–Ammonium Hydroxide (17:2:1)	Instant Thin-Layer Silica Gel Sheet: *n*-Butanol–Acetic Acid–Water (35:3:10)	Instant Thin-Layer Silica Gel Sheet: *n*-Butanol–*n*-Butyl Ether–Ammonium Hydroxide (25:70:2)	Silica Gel Precoated Plates, Ethyl Acetate–Methanol–Ammonium Hydroxide (17:2:1)
Codeine	98	98	70	60
Morphine	97	98	62	34
Morphine *N*-oxide	40	90	23	3
Norcodeine	97	98	64	34
Normorphine	85	97	50	15
Morphine-3-ethereal sulfate	47	67	19	3
Morphine glucuronide	0	40	0	0
Pseudomorphine	10–15	30	0	1
Morphine-*N*-methyl iodide	0	75	3	0
Nalorphine	98	90	81	50
Nicotine and its metabolites[a]	0, 8, 27, 50 70, 90	90, 96	0–23, 65–80	4, 8, 34 64, 83

[a] Two hundred milliliters urine from a heavy cigarette smoker (two packages per day) was passed through XAD-2 resin, which was then washed with water and eluted with methanol. The methanolic eluate was concentrated to 3 ml., and 50 μl. was spotted.

Table II—Retention Time (Minutes) of Morphine and Its Metabolites and Congeners

Compounds	Column 1, 100–120 Varaport 205°	Column 1, 100–120 Varaport 215°	Column 2 (0.91-m.), 60–80 Gas Chrom Q 205°	Column 2 (0.91-m.), 60–80 Gas Chrom Q 215°	Column 3 (1.82-m.), 60–80 Gas Chrom Q 200°	Column 3 (1.82-m.), 60–80 Gas Chrom Q 230°	Column 3 (1.82-m.), 60–80 Gas Chrom Q 250°
Trimethylsilyl							
-codeine	—	7	—	7.7	45.4	13.4	6.5
-morphine	—	6.0	—	6.7	34.5	10.5	5.7
-morphine *N*-oxide	—	6.0	—	6.7	34.5	10.5	5.7
-morphine-*N*-methyl iodide[a]	—	4.0	—	4.7	—	12.0	5.8
-norcodeine	—	6.2	—	8.2	—	—	—
-normorphine	—	6.3	—	10.3	—	19	8.0
-pseudomorphine[b]	—	8.5	—	8.3	41.3	12.5	6.5
Acetyl	—	—	—	—	—	—	—
-codeine	—	6.0	—	13.5	—	—	—
-morphine	—	8.0	—	24	—	—	—
-morphine *N*-oxide	—	7.8	—	23.2	—	—	—
-morphine-*N*-methyl iodide	—	8.0	—	19	—	—	—
-norcodeine	—	19.7	—	13.5	—	—	—
-normorphine	—	27.5	—	19 (250°)	—	—	—
Trifluoroacetyl							
-codeine	5.2	3	7.7	4	—	—	—
-morphine	4.4	2.8	5.1	3.5	15	—	—
-morphine *N*-oxide	6.0	4.5	7.0	—	20	—	—
-norcodeine	7.5	—	13.0	—	—	—	—
-normorphine	7.1	—	8.7	—	—	—	—
-pseudomorphine[c]	53[c]	—	—	—	—	—	—

[a] Morphine-*N*-methyl iodide gave two peaks which may arise from thermal decomposition. [b] Trimethylsilyl-pseudomorphine did not emerge from the column at 290° after 1 hr. [c] The retention time for trifluoroacetyl-pseudomorphine was obtained at a column temperature of 290°.

butyl ether–ammonium hydroxide (25:70:2). Morphine and one spot of the resin[8] extract of a urine from a cigarette smoker had approximately the same R_f value on the silica gel plates. The two spots could be distinguished by their color after spraying with iodoplatinate reagent. Morphine had a blue-purplish color and the nicotine metabolite was gray. Also, the nicotine metabolite moved slightly ahead of the morphine.

Conjugated morphine metabolites, *i.e.*, morphine-3-glucuronide and morphine-3-ethereal sulfate, could be separated from the free drugs on the instant silica gel sheets developed either with ethyl acetate–methanol–ammonium hydroxide (28% ammonia) (17:2:1) or with *n*-butanol–acetic acid–water (35:3:10).

GLC Separation of Morphine and Its Metabolites and Congeners—The GLC data for morphine and its metabolites are presented in Table II. An earlier report (23) that silyl derivatives of norcodeine and normorphine were retained in the 3% SE-30 column (Column 1) even at 250° was in error. The error, which might be due to the incompleteness of the silylation of these compounds or to the low concentrations of these compounds applied, should be corrected with the present data. Since the trimethylsilyl derivatives of morphine and its metabolites could not be completely separated with the 0.91-m. (3-ft.) 3% OV-17 Column 2, the 1.82-m. (6-ft.) 3% OV-17 Column 3, or Column 1, acetyl derivatives of morphine and its free metabolites were prepared and examined with these columns. Good separations of these compounds, except morphine *N*-oxide and morphine, were obtained with Columns 1–3.

These data agree well with earlier data (25), except the retention times were longer in the present study. The difference in retention times was apparently due to the different size of supports used. The retention times of acetylnormorphine and acetylnorcodeine on Column 1 at 215° was too long for practical use, and the curves tailed. With temperature programming, the retention times of these compounds were considerably shortened and the peaks sharpened. The retention times of acetyl derivatives of codeine, morphine, norcodeine, and normorphine on Column 1, using temperature programming from 205 to 250° at 2°/min., were 7.3, 9.5, 16.4, and 18.2 min., respectively. Since the retention times of the acetyl derivatives of normorphine and norcodeine on Columns 1–3 were quite long and the curves tailed, trifluoroacetyl derivatives were prepared and examined on these columns.

The trifluoroacetyl derivatives of morphine and its free metabolites, except morphine *N*-oxide and normorphine, could be separated very well with Column 1 and gave sharp peaks (Fig. 1B).

[8] XAD-2.

Table III—Retention Time (Minutes) of Chemicals Used as Internal Standards for Quantitative Determination of Morphine and Its Metabolites

Compounds	Column 1, 100–120 Varaport			Column 2 (0.91-m.), 60–80 Gas Chrom Q			Column 3 (1.82-m.), 60–80 Gas Chrom Q		
	200°	205°	215°	200°	205°	215°	200°	230°	250°
Trifluoroacetyl-nalorphine	8.1	6.9	4.8	9.8	7.1	5.1	—	8.0	4.1
Tetraphenylethylene	10.6	8.5	6.1	19	15	10.2	>37	16.7	8.0
Cholestane	35	23.5	15.0	23.5	18.2	12	>30	22.0	10.5
Docosane	4.3	3.4	2.0	2.2	1.6	0.9	5.5	2.0	1.0
Tetracosane	8.2	6.6	4.5	3.6	3.0	2.2	11.8	4.2	1.8
Octradosane	33	25.5	17.4	16.0	12.8	8.0	>30	13.0	5.8
Methylarachidate	6.5	4.7	3.5	5.3	3.5	2.4	13.0	4.1	2.1
Methylbehanate	12.4	9.6	6.4	8.9	6.9	4.7	27.3	7.8	7.0

These compounds, except codeine and morphine *N*-oxide, could also be completely separated with Column 2 (Fig. 1A). The trifluoroacetyl-normorphine and trifluoroacetyl-norcodeine on Columns 2 and 3 showed some tailing. The retention time of trifluoroacetyl-pseudomorphine, prepared by heating pseudomorphine with trifluoroacetic anhydride for 2 hr., was 53 min. on Column 1 at 290°. Trifluoroacetyl-pseudomorphine did not emerge from Columns 2 and 3 at 290° for 2 hr. Trimethylsilyl-pseudomorphine, prepared by heating the compound with 0.1 ml. acetonitrile and 0.1 ml. bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane at 60–70° for 2 hr., did not emerge from Columns 1–3 at 290° for 2 hr. Silylation or acetylation, either with trifluoroacetic anhydride or acetic anhydride, could be accelerated by heating the mixture in a screw-capped sealed tube at 60–70° for 0.5 hr. Acetylation with trifluoroacetic anhydride could not be completed without heating for at least 24 hr. During the preparation of this paper, a similar procedure for acetylation of codeine and its metabolites with trifluoroacetic anhydride appeared in the literature (24).

The trifluoroacetyl derivatives of morphine and its metabolites were quite unstable and lost their trifluoroacetyl moiety on standing in air or in ethyl acetate solution. Therefore, the mixture should be kept sealed in the trifluoroacetic anhydride medium until a few hours prior to injection. After removing the excess trifluoroacetic anhydride and dissolving the residue with ethyl acetate, the solution should be injected within a few hours. A low sensitivity was obtained when the solution was stored overnight. Trifluoroacetyl-morphine *N*-oxide was very unstable in ethyl acetate; the solution became yellow within 1 hr.

The retention times of seven other chemicals, which are commonly used as internal standards for quantitative determination of morphine and its metabolites and congeners by GLC, were determined on Columns 1–3 (Table III). The retention times varied with changes in temperature and type of column, but the basic pattern remained the same. Nalorphine has been suggested as an internal standard to be added to the urine for determination of morphine (25). The retention time of trifluoroacetyl-nalorphine on Column 1 interfered with the retention time of trifluoroacetyl-norcodeine; on Column 2, it interfered with that of trifluoroacetyl-codeine. Among the chemicals examined, tetraphenylethylene was the best internal standard for quantitative determination of trifluoroacetyl-morphine or trifluoroacetyl-codeine and their metabolites on Column 1.

Urinary disposition of narcotics is being currently investigated with the described TLC and GLC procedure. Quantitative determinations of urinary disposition of morphine in morphine-dependent subjects have been reported elsewhere (26).

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