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Detection, isolation and characterization of normorphine and norcodeine as morphine metabolites in man

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Normorphine and norcodeine were identified by two-dimensional thin-layer chromatography (t.l.c.) as human urinary morphine metabolites from 24 h urine samples of three patients who had received 190–210 mg morphine sulphate intravenously and from one patient who had chronically received 220 mg morphine sulphate orally per day. Both metabolites were isolated by preparative t.l.c. Comparison of the isolated norcodeine and normorphine with their respective authentic compounds, prepared by chemical synthesis, showed identical melting points, mass fractionation patterns, and chemical ionization spectra, confirming t.l.c. identification.

In the screening of the urine specimens of heroin addicts in this laboratory, spots that corresponded in R_F and iodoplatinate colour to normorphine and norcodeine were frequently observed on the thin-layer chromatography (t.l.c.) plates. Heroin is rapidly hydrolysed to 6-monoacetylmorphine and then to morphine *in vivo* (Way, Young & Kemp, 1965) whilst morphine is excreted as morphine 3-glucuronide and free morphine. *N*-Demethylation of morphine to normorphine has been reported to occur in animals after morphine administration (Milthers, 1961, 1962) and the formation of codeine from morphine in human addicts has been reported (Boerner & Abbott, 1973).

Recently, Yeh (1973), using t.l.c., detected normorphine and normorphine conjugates in the urine of volunteers given a single oral dose of morphine sulphate. We have been able to isolate from urine and characterize by chemical ionization and electron impact mass spectrometry (ms) both normorphine and norcodeine as morphine metabolites. These metabolites were present after a large single dose and after chronic administration of morphine.

MATERIALS AND METHODS

Materials

Sample collection. Three patients under morphine for cardiac surgery each received 190–210 mg morphine sulphate intravenously over 3 h. A fourth patient received morphine sulphate chronically, 220 mg orally per day. The morphine used was analysed by two-dimensional t.l.c. and by electron impact ms and found to contain only trace amounts of normorphine. Codeine was not detected. (Commercial morphine can contain up to 1·5% normorphine; Miller, Jolles & Rapaport, 1973.)

Urine samples were collected over 24 h from each patient during the intra- and postoperative period in the acute treatment patients and during days 20–23 in the chronic treatment patient. Before analysis all samples were adjusted to pH 6 with 0·1N HCl, if necessary, and stored at −25°. No preservatives were added.

Reference substances. Norcodeine was synthesized from codeine according to Braun, Kruber & August (1914). The norcodeine free base was recrystallized from hot ethanol and further purified on preparative t.l.c. and then, after visualization (ultraviolet), eluted from the silica with hot methanol. Norcodeine was obtained as free base mp 185°, (m.p. lit. The Merck Index, 1968, 185°). No impurities were detected by t.l.c. and electron impact ms.

Samples of *N*-desmethylmorphine (normorphine) were generously donated by Drs. D. Cluet, Brooklyn, New York, and H. Rapaport, Berkeley, California. Normorphine was also prepared according to the method of Braun & others (1914) and purified as for norcodeine. Normorphine was obtained as free base, 1/2 mol crystal methanol, m.p. 272–273°, (lit. The Merck Index, 1968, 272–273°). The yield was poor. No impurities were detected by two-dimensional t.l.c. and electron impact ms.

Reagents. All reagents were American Chemical Standard analytical grade; all solvents were spectrograde quality. β-Glucuronidase-arylsulfatase was obtained from Calbiochem, San Diego, California, as the glycerine-stabilized liquid concentrate, containing 200 000 Fishman units and 20 000 Whitehead units ml^{-1} solution at 37° and pH 4·5. T.l.c. solvent system I consisted of ethanol–benzene–1,4-dioxane–concentrated aqueous ammonia (50 : 40 : 5 : 5), and solvent system II consisted of 1,4-dioxane–chloroform–ethyl acetate–concentrated aqueous ammonia (60 : 25 : 10 : 5).

Preparation of urine samples. The 24 h urine sample (480–1300 ml) from each patient was analysed as follows: ammonium chloride (20 mg ml^{-1}), 1000 Fishman units of β-glucuronidase, and 100 Whitehead units of arylsulfatase (at 37°) per ml of urine were added to each sample aliquot. The urine was adjusted with 5 N HCl to pH 4·5 and incubated for 20 h in a water-bath at 40–43°. The urine was then cooled to 10°, adjusted with 5 N NaOH to pH 10, and extracted three times with 300 ml benzene. After extraction, the aqueous phase was analysed immediately for normorphine which was isolated by saturation of the aqueous phase with NaCl and adjustment with HCl to pH 8·9 (Milthers, 1961, 1962). The solution was extracted four times with 50% by volume of chloroform–isoamyl alcohol (3 : 1). Emulsions were broken by centrifugation.

The combined benzene extracts and the combined chloroform-isoamyl alcohol extracts were each washed separately with 100 ml saturated NaCl solution and dried over anhydrous magnesium sulphate. To prevent decomposition of the opiate bases, 1 drop of glacial acetic acid was added and the solution was dried under vacuum without heat. The remaining residue was dissolved in methanol–acetone (1 : 1), applied to preparative chromatoplates, and chromatographed using t.l.c. and system II. The zones of codeine, norcodeine, morphine and normorphine and the metabolites were isolated as for the reference substances. An aliquot of the residue of the benzene extraction and an aliquot of the residue of the chloroform–isoamyl alcohol extraction were each chromatographed in two dimensions using solvent systems I and II. The resultant R_F values were: normorphine 0·19 and 0·05, norcodeine 0·27 and 0·19, morphine 0·44 and 0·20, and codeine 0·56 and 0·41 after a 10 mm run of the two solvent systems at 90° to each other on the t.l.c. plates described below.

The addition of acetic acid to the normorphine extract resulted in partial acetylation of the normorphine of approximately 5–10% as estimated on t.l.c. in preliminary tests with the authentic compound (Stahl, 1969), which did not significantly interfere with further analytical procedures. Omission of the acetic acid, however, resulted in

extensive decomposition of normorphine and morphine, yielding tarry breakdown products that could not be characterized.

Thin-layer chromatography. The t.l.c. separations were made using 20 × 20 cm glass plates coated with 0·25 mm silica gel GF-254 type 60 according to Stahl (1969). Preparative t.l.c. was on identical plates using a 1·2 mm silica coating. Two-dimensional t.l.c. was performed using solvent system I as the first and system II as the second mobile phase. The solvent front was 10 cm in both directions. The spray reagent was potassium iodoplatinate solution, prepared according to Hilf, Castano & Lightbourn (1959); it had a sensitivity limit after a two-dimensional run of 0·3 μg for morphine, 0·5 μg for norcodeine and 0·8 μg for normorphine.

Mass spectrometry. All reference substances and isolated metabolites were analysed by high resolution electron impact ms on a GEC-AEI-MS-902 mass spectrometer at 70 eV and 220° using a Logos computer program and data printout as described by Smith, Olsen & others (1971). The chemical ionization low resolution mass spectra were obtained on an AEI-MS-902 mass spectrometer at 70 eV and 220° using isobutane at 0·7 mm Hg.

Proton magnetic resonance. To ascertain further the structures of the isolated metabolites, pmr spectra of the authentic nor-compounds were obtained on a Gelco 60 MHz. Tetramethylsilane was used as the internal standard. Deuterochloroform was used as the solvent for norcodeine and deutero-dimethyl sulphoxide as the solvent for normorphine.

RESULTS

Normorphine and norcodeine were isolated from the urine of all 4 patients using preparative techniques and were separated and purified on t.l.c. and detected and characterized from urine extracts by two-dimensional t.l.c. The pmr spectrum of the isolated normorphine was identical to that from authentic normorphine. Insufficient norcodeine was isolated for pmr spectrometry.

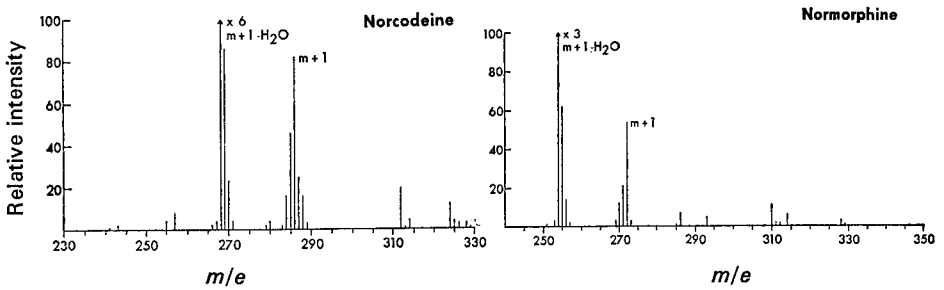

FIG. 1. Isobutane chemical ionization mass spectra obtained from the isolated urinary metabolites.

The chemical ionization mass spectra of the isolated metabolites corresponded well with those known for *N*-methyl analogues (Fales, Milne & Vestal, 1969; Milne, Fales & Axenrod, 1971) and were characterized by strong quasi-molecular ions, M + 1 and M + 1-H_2O, which are *m/e* 286 and 268 for norcodeine and *m/e* 272 and 254 for normorphine. The ions observed at *m/e* 324 (Fig. 1, norcodeine) and *m/e* 310 (Fig. 1, normorphine) may represent quasimolecular ions of M + 39 formed by the reactant

gas and the norcompounds. The formation of such ions has been reported by Foltz (1972). Small impurities of the monoacetylnorcompounds might have given rise to the peaks at *m/e* 328 and 314. The high resolution electron impact mass spectra were recomputed and simplified by the Logos system (Smith & others, 1971) and are presented as recharted low resolution mass spectra. The spectra obtained from the isolated norcodeine and normorphine (Fig. 2) corresponded in their major fractionation products to those observed for the *N*-methyl analogues (Wheeler, Kinstle & Rinehart, 1967).

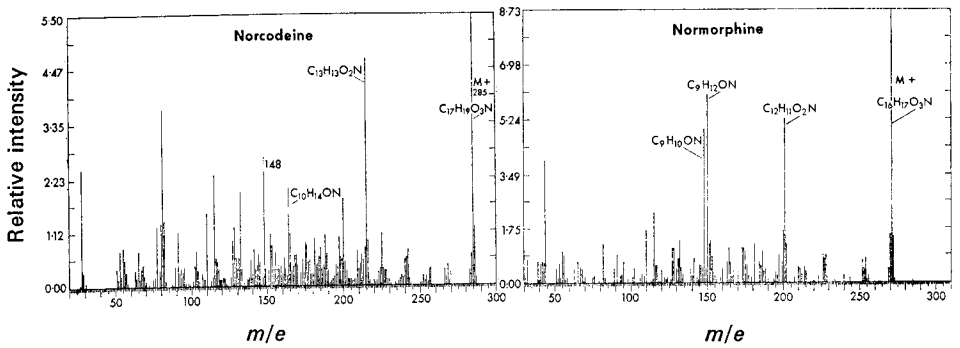

FIG. 2. High resolution electron impact mass spectra of isolated metabolites.

The recovery of normorphine from urine varied, but for all subjects ranged from 3–6% of the administered morphine dose. These values were corrected for losses and decomposition during work-up and have taken into account the poor solubility of normorphine in water-immiscible solvents. Preliminary blank recoveries with authentic normorphine added to the urine of normal subjects showed the recovery to be $<65\%$ at pH 8·9 in chloroform–isoamyl alcohol (3 : 1), after four extractions, with a 1 : 2 ratio by volume of extraction solvent to aqueous phase.

The amounts of normorphine and norcodeine in the urine of the patient chronically ingesting large doses of morphine were estimated (Stahl, 1969) to be approximately 5% normorphine and $>0{\cdot}1\%$ norcodeine relative to the administered morphine dose.

DISCUSSION

The preparative isolation of normorphine and norcodeine from the urine of patients given morphine with subsequent confirmation by chemical ionization and electron impact ms has now been accomplished. The results substantiate previous observations made using t.l.c. techniques (Yeh, 1973; Boerner & Abbott, 1973). The presence of normorphine and norcodeine in urine of human subjects receiving a single large dose or chronic doses of morphine suggests that morphine is metabolized to a limited extent to normorphine and to codeine and norcodeine (Fig. 3).

The formation of normorphine and its excretion into urine seem to occur in all patients after morphine administration. The difficulty in detecting normorphine may be due to its instability in both acidic and alkaline media, to its poor solubility in water-immiscible solvent systems and/or to its limited sensitivity to potassium iodoplatinate, the spray reagent commonly used for alkaloids in t.l.c. In addition, the small urine aliquots of 10–20 ml usually used for opiate analysis may not contain

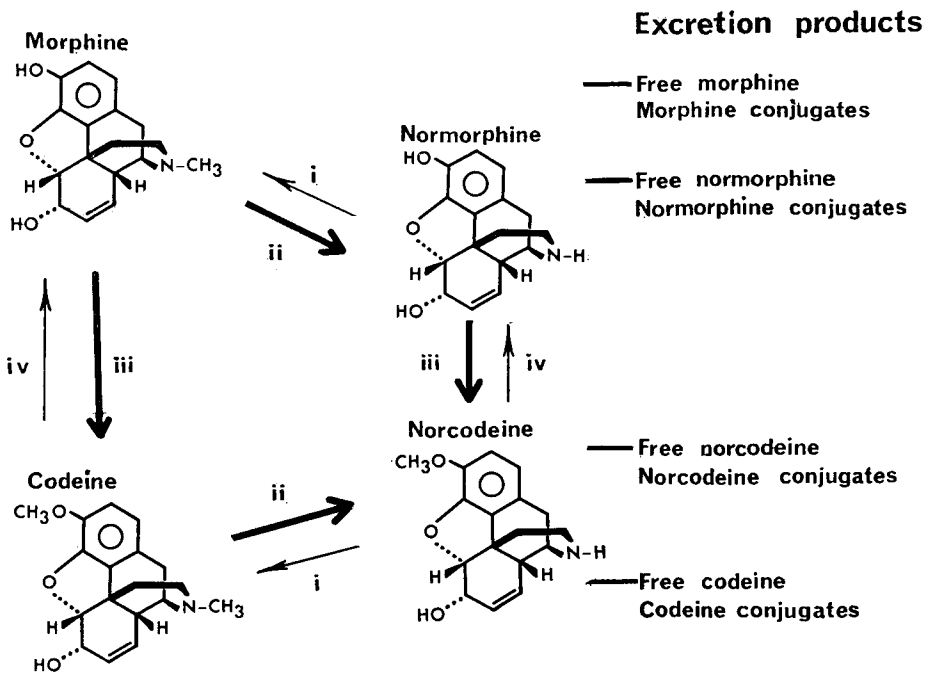

FIG. 3. Possible metabolic pathways of opiates in man (i = *N*-methylation; ii = *N*-dimethylation; iii = *O*-methylation; iv = *O*-demethylation.

sufficient metabolite to distinguish normorphine from other compounds in the urine.

From the results obtained from the patient who received morphine chronically, it would seem that quantitative differences in the metabolism of morphine exist in the morphine-tolerant subject as opposed to the acutely treated subjects since that patient had higher urinary levels of codeine and norcodeine than the patients who received a single dose of morphine. Thus *O*-methylation appears to be increased in morphine-tolerance. Since *N*-demethylation of codeine to norcodeine is known to occur after codeine administration (Adler, Fujimoto & others, 1955) and since normorphine is also formed after codeine administration (Ebbighausen, Mowat & Vestergaard, 1973), it seems probable that these reactions take place via both *O*-methylation and *N*-methylation and that they may proceed concomitantly in the metabolic sequence depicted in Fig. 3.

Norcodeine was detected and recovered in quantities proportional to the formation of codeine. This has been observed previously in studies of codeine metabolism (Adler & others, 1955). Codeine formation (Börner & Abbott, 1973) and consequent norcodeine formation, however, seem to appear after prolonged heroin abuse. It is unclear whether this increase is due to substrate induction or due to other changes in hepatic functions frequently observed in opiate addicts but probably unrelated to hepatotoxicity of opiates (Potter, Cohen & Norris, 1960; Gorodetzky, Sapira & others, 1968).

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