ical technique employed for dissolution studies, one must verify that the excipients present in the formulations indeed do not interfere with the titration assay. The titration method cannot be employed in studying dissolution of capsule formulations, since the acidic nature of gelatin capsules causes overlap with the titration assay of the drug. In the automated spectrophotometric method, the dissolution fluid has to be filtered and then externally circulated through a photometric cell. Therefore, clogging of the filter screen and the lag time involved between the actual dissolution of the drug and its spectrophotometric measurement are the obvious problems encountered with this method. In the titration technique, however, these problems are nonexistent because the dissolution fluid does not have to be filtered or externally circulated.

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NOTES

Metabolites of Naloxone in Human Urine

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Abstract □ Naloxone, 7,8-dihydro-14-hydroxynormorphinone, and *N*-allyl-7,8-dihydro-14-hydroxynormorphine have been identified in a human urine specimen after naloxone administration. The metabolites were isolated, after hydrolysis with glusulase, by column chromatography and identified by TLC and spectrofluorimetry. These findings indicate that both *N*-dealkylation and the reduction of the 6-keto group of naloxone as well as glucuronide formation occur in man. Evidence is also presented indicating that *N*-allyl-7,8-dihydro-14-hydroxynormorphine formation occurs in rabbits as well as in man and the chicken.

Keyphrases □ Naloxone, metabolites—determination in human urine □ 7,8-Dihydro-14-hydroxynormorphinone, naloxone, and *N*-allyl-7,8-dihydro-14-hydroxynormorphine—isolated and identified as metabolites of naloxone in human urine □ Spectrofluorimetry—identification, naloxone metabolites □ TLC—identification, naloxone metabolites

Naloxone (*N*-allyl-7,8-dihydro-14-hydroxynormorphinone) is a potent narcotic antagonist in laboratory animals (1, 2) and in man (3). Fujimoto's (4) isolation of the *N*-allyl-7,8-dihydro-14-hydroxynormorphine-3-glucuronide metabolite of naloxone in chicken urine aroused speculation concerning its occurrence as a human metabolite. In rabbit (4) and in man (5), naloxone glucuronide has been reported as the sole metabolite of naloxone.

In the present study, an isolation procedure developed by Fujimoto and his coworkers (4–6) was used to separate the metabolites of naloxone. These were identified as the glucuronide of naloxone, 7,8-dihydro-14-hydroxynormorphinone (EN-3169), and *N*-allyl-7,8-dihydro-14-hydroxynormorphine (EN-2265).

EXPERIMENTAL

Approximately 2 l. of urine from a pooled 2-week urine collection was obtained from a patient. The urine was preserved under toluene and refrigerated. The patient was participating in a study of naloxone in the treatment of opiate dependence and was receiving between 1.0 and 1.8 g. of naloxone in single, daily oral doses during the 2-week collection period.

Column Chromatography—Metabolites were isolated from urine on a resin column[1] (5). Prior to column chromatography, 10 ml. of urine was adjusted to pH 5.3 with acetic acid and incubated overnight at 37° with 0.2 ml. of glusulase[2], an extract of *Helix pomatia* intestine which contains glucuronidase and sulfatase activity. The glusulase-treated urine was applied to a 2.5 × 10-cm. column of the resin. This process was followed by 50 ml. of distilled water and then 300 ml. of absolute methanol. The urine and water effluents were discarded. After discarding the first 10 ml. of methanol effluent, 250 ml. was collected. The methanol eluate was evaporated to dryness on a rotary evaporator[3] and redissolved in 0.2 ml. of methanol.

TLC—Five microliters of the concentrated methanol eluates was applied to a precoated silica gel TLC plate[4]. Controls and standards treated in a manner identical with that already described were also applied to the plate. Plates were developed in two solvent systems: A, chloroform–methanol–acetic acid (100:60:2 v/v); and B, chloroform–dioxane–ethyl acetate–concentrated ammonia (25:60:10:2.5 v/v). The solvents were used singly for developing

[1] Amberlite XAD-2.
[2] Endo Laboratories, Garden City, N. Y.
[3] Buchler.
[4] E. Merck ag. E. M. Reagents Division, Brinkmann Instruments.

Table I—R_f Values of Naloxone and Its Metabolites

Compound	R_f in Solvent System A[a]	R_f in Solvent System B[b]
Naloxone	0.65	0.35
EN-2265	0.40	0.22
EN-3169	0.29	0.04

[a] Solvent A: chloroform–methanol–acetic acid (100:60:2 v/v). [b] Solvent B: chloroform–dioxane–ethyl acetate–concentrated ammonia (25:60:10:2.5 v/v).

in one direction and in combination (Solvent A followed by Solvent B) for two-dimensional TLC.

Spray Reagents—Detection of the metabolites on TLC plates was achieved with iodoplatinate spray reagent (7), ferri-ferrocyanide reagent (8), and Dragendorff reagent oversprayed with 5% aqueous sodium nitrite (9). Spraying with iodoplatinate resulted in characteristic blue to purple spots on a pink background. The Dragendorff–nitrite spray revealed the metabolites as brown spots on a yellow background, and the ferri-ferrocyanide reagent oxidized suitable substrates to fluorescent derivatives which were observed as blue spots on a purple background under UV light.

Spectrofluorimetry—A spectrophotofluorometer[5] was used to obtain the fluorescence excitation and emission spectra of EN-2265 after reaction with ferri-ferrocyanide reagent (10). Pure EN-2265 was oxidized to "pseudo-EN-2265," 2,2′-bi(*N*-allyl-7,8-dihydro-14-hydroxynormorphine), with ferri-ferrocyanide reagent, and the spectra were obtained for comparison with material eluted from TLC plates after spraying with ferri-ferrocyanide spray reagent. The eluting agent was 1 ml. of 0.1 *M* sodium pyrophosphate adjusted to pH 8.5 with 1 *N* hydrochloric acid. The eluate was transferred to a microcell for fluorometric scanning.

RESULTS

TLC of the glusulase-hydrolyzed urine sample in the two solvent systems, both singly and two dimensionally, resulted in the detection of three spots. These spots were visualized with both iodoplatinate and Dragendorff–nitrite reagents and coincided with naloxone, EN-2265 (the reduced naloxone), and EN-3169 (the dealkylated naloxone). The spots coinciding with EN-2265 and standard EN-2265 were the only ones that fluoresced under UV light after being sprayed with ferri-ferrocyanide reagent. Without glusulase hydrolysis, iodoplatinate reagent revealed only one spot near the origin. This spot, undetectable with the other spray reagents, probably consists of the conjugates of naloxone and its metabolites. The R_f values of naloxone and its metabolites are presented in Table I.

Two-dimensional TLC was necessary to obtain adequate separation of EN-2265 from other substances for spectrofluorimetry. The fluorescent spot obtained after two-dimensional TLC and spraying with ferri-ferrocyanide was eluted. The fluorescence excitation and emission spectra of the metabolite were identical with those of pure EN-2265, which has an excitation maximum of 330 nm. and an emission maximum of 430 nm. (both maxima uncorrected).

DISCUSSION

Evidence has been presented for the occurrence of several conjugated metabolites of naloxone in the urine of a human subject. The substances were isolated from urine, after hydrolysis with glusulase, and identified by TLC as naloxone, 7,8-dihydro-14-hydroxynormorphinone (EN-3169), and *N*-allyl-7,8-dihydro-14-hydroxynormorphine (EN-2265). The identification of the latter compound was verified by its fluorescence emission and excitation spectra. A free phenolic hydroxy group in the 3-position and a free 6-hydroxyl group are necessary for formation of a fluorescent derivative when oxidizing with ferri-ferrocyanide reagent.

The requirement for hydrolysis before the free bases are detectable and the well-documented conversion of similar drugs to glucuronides indicate that these conjugated metabolites are normally present as glucuronides. Fujimoto (4) reported the presence of EN-2265 and of naloxone glucuronides in the urine of chickens given naloxone orally. Naloxone 3-glucuronide was also isolated from human urine (5). Since glusulase contains sulfatase as well as glucuronidase, the possibility of the occurrence of sulfate ether conjugates exists, but this has not been demonstrated.

Nalorphine and structurally analogous drugs are enzymatically *N*-dealkylated by liver microsomes (11, 12). The *N*-dealkylated metabolite of naloxone, EN-3169, was identified in human urine, in this study, by comparison with a standard on TLC.

Although Fujimoto (5) did not rule out the formation of EN-2265 from naloxone in man, he reported detecting nothing corresponding to EN-2265 after TLC of human urine extracts. To correlate data from this laboratory with his, Fujimoto supplied us with the glucuronides isolated from the urine of rabbits and chickens which had been treated orally with naloxone. These glucuronides were hydrolyzed and chromatographed as described in the *Experimental* section. While Fujimoto and Haarsted (6) reported the formation of EN-2265 in chickens but not in rabbits, we found it to be present in the glucuronides of both species. Although EN-2265 occurs in rabbits at a much lower concentration than in chickens, it was detectable with Dragendorff–nitrite and ferri-ferrocyanide sprays.

These findings indicate that EN-2265 is a metabolite of naloxone in rabbit, chicken, and man, but that its lower concentration in rabbit and man requires more sensitive detection methods than iodoplatinate spray reagent. The use of glusulase rather than acid hydrolysis and the TLC methodology provided the necessary sensitivity.

[5] Baird-Atomic, model SF-1.

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