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Note

Determination of morphine, morphine-3-glucuronide and (tentatively) morphine-6-glucuronide in plasma and urine using ion-pair high-performance liquid chromatography

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Morphine is metabolized in man primarily through conjugation with uridine diphosphoglucuronic acid in the 3-position. This conjugate is the major metabolite of morphine in several mammals [1] and it accounts for about 54—74% of the excretion products in man [2]. A minor portion is glucuronidated in the 6-position or demethylated to normorphine [3]. While investigating the clinical pharmacology of morphine in cancer patients with severe pain [4], a need arose to develop a simple analytical method for morphine and its metabolites in plasma and urine. We now present such a method for the simultaneous analysis of morphine, morphine-3-glucuronide and normorphine by high-performance liquid chromatography (HPLC) using ion-pair formation. A metabolite which is tentatively identified as the morphine-6-glucuronide is also coanalyzed in this method. The chromatographic system may also be used for the analysis of the morphine congeners codeine, ethylmorphine and heroin (diamorphine).

EXPERIMENTAL

Materials

Morphine-3-glucuronide and normorphine were generously supplied by the National Institute of Drug Abuse, Bethesda, MD, U.S.A. 1-Dodecyl sulphate (sodium salt) was chromatography grade (Regis Chemicals, Morton Grove, IL, U.S.A.). Acetonitrile was HPLC grade. All other chemicals were analytical reagents. The water used was glass-distilled.

Before use the Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were treated with 5 ml of methanol, 3 ml of a 10% acetonitrile solution in 10 m*M* phosphate buffer (pH 2.1), and 5 ml of water.

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Apparatus

The chromatographic equipment consisted of a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a 7120 injector (Rheodyne, Berkeley, CA, U.S.A.) with a 400-μl loop, an Ultrasphere ODS 150 × 4.6 mm reversed-phase column (5-μm particles; Altex, Berkeley, CA, U.S.A.) and a Spectromonitor III variable-wavelength UV detector (Laboratory Data Control).

Chromatographic conditions

The eluent was a 10 m*M* sodium dihydrogen phosphate buffer pH 2.1 (adjusted with phosphoric acid) containing 1 m*M* dodecyl sulphate and 26% acetonitrile. The flow-rate was 1.5 ml/min. The temperature was ambient and the detector wavelength was set at 210 nm.

Sample purification

One millilitre of plasma or urine (the urine samples normally had to be diluted) was mixed with 3 ml of 0.5 *M* ammonium sulphate adjusted to pH 9.3 with ammonia. This sample was passed through the Sep-Pak C_{18} cartridge. The cartridge was washed with 20 ml of 5 m*M* ammonium sulphate adjusted to pH 9.3 with ammonia, and 0.5 ml of distilled water. Morphine and its metabolites were eluted with 3.0 ml of a 10% acetonitrile solution in 10 m*M* phosphate buffer pH 2.1. The eluate was mixed with 3 ml of the 0.5 *M* ammonium buffer and treated on a second Sep-Pak C_{18} cartridge in the same way as on the first one. Part of the eluate (400 μl) was injected on the column.

RESULTS AND DISCUSSION

The separation of opium alkaloids by reversed-phase ion-pair HPLC has been described earlier [5,6]. A method similar to that used by Kubiak and Munson [6], but using 0.1 *M* phosphate buffer pH 3.3, 36% acetonitrile, and 5 m*M* dodecyl sulphate as pairing ion, was initially tested in our laboratory. This system provided a good separation of morphine from its congeners (Table I).

TABLE I

RETENTION TIMES FOR OPIUM ALKALOIDS

For conditions, see text.

Compound	Retention time (min)
Morphine	3.5
Codeine	5.8
Ethylmorphine	8.9
Heroin (diamorphine)	18.7

The detector wavelength was set at 210 nm to obtain the best possible response for morphine. For the determination of morphine-3-glucuronide it was necessary to decrease the acetonitrile concentration to 26% to improve the separation. The pH was adjusted to 2.1 in order to suppress the ionization of the glucuronic acid group ($pK_a \approx 3.2$). The separation between morphine-3-

glucuronide and impurities was improved by changing the dodecyl sulphate concentration to 1 m*M*.

Since morphine-3-glucuronide is a very polar compound, conventional organic extraction from an aqueous solution is difficult. We have found the Sep-Pak C_{18} cartridge to be a convenient tool for the purification of plasma and urine samples. A second Sep-Pak purification step was found to further exclude impurities. This was particularly important for the analysis of low morphine concentrations. Eighty-four per cent of morphine at a concentration of 100 ng/ml and 90% of the morphine-3-glucuronide at a concentration of 1000 ng/ml were recovered after the two-step purification on the Sep-Pak cartridges.

Standard curves were obtained by analysis of plasma spiked with morphine and morphine-3-glucuronide. The concentration range was 20—100 ng/ml for morphine, and 200—1000 ng/ml for morphine-3-glucuronide. The peak areas (peak height × peak width at half height) in mm^2 at 0.01 absorbance units full scale (a.u.f.s.) on the recorder were determined. The standard curves were linear for both morphine ($Y = 1.310X - 0.084$; $r = 0.9996$) and morphine-3-glucuronide ($Y = 0.992X + 0.740$; $r = 0.9999$). The coefficient of variation for the analysis of morphine in plasma was 7.6% at a level of 22 ng/ml ($n = 6$) and 3.8% at a level of 223 ng/ml ($n = 5$). The coefficient of variation for the analysis of morphine-3-glucuronide in plasma was 1.3% and 2.7% at a level of 200 and 2.100 ng/ml, respectively ($n = 5$). Minimum detectable amounts for morphine and morphine-3-glucuronide were 5 ng/ml. Chromatograms of blank plasma and of plasma spiked with morphine-3-glucuronide, normorphine and morphine are shown in Fig. 1. Chromatograms of plasma and urine from morphine-treated cancer patients are shown in Figs. 2 and 3.

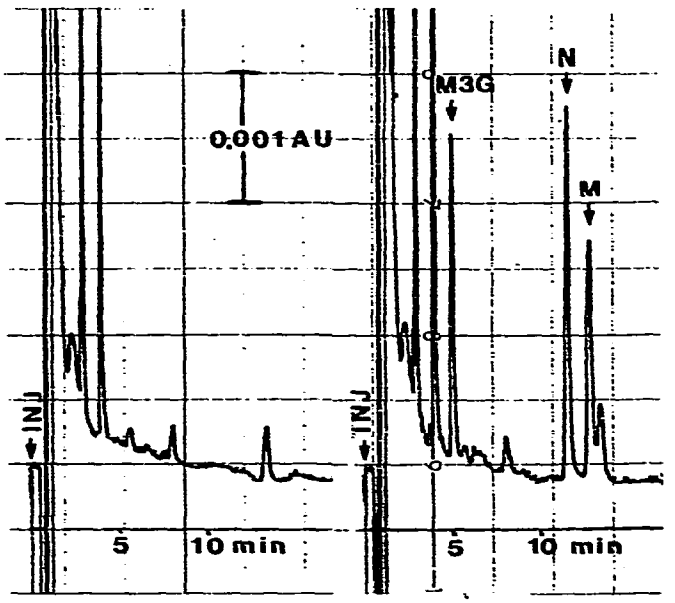

Fig. 1. Chromatograms of blank plasma, and of blank plasma spiked with about 100 ng/ml each of morphine-3-glucuronide (M3G), normorphine (N) and morphine (M).

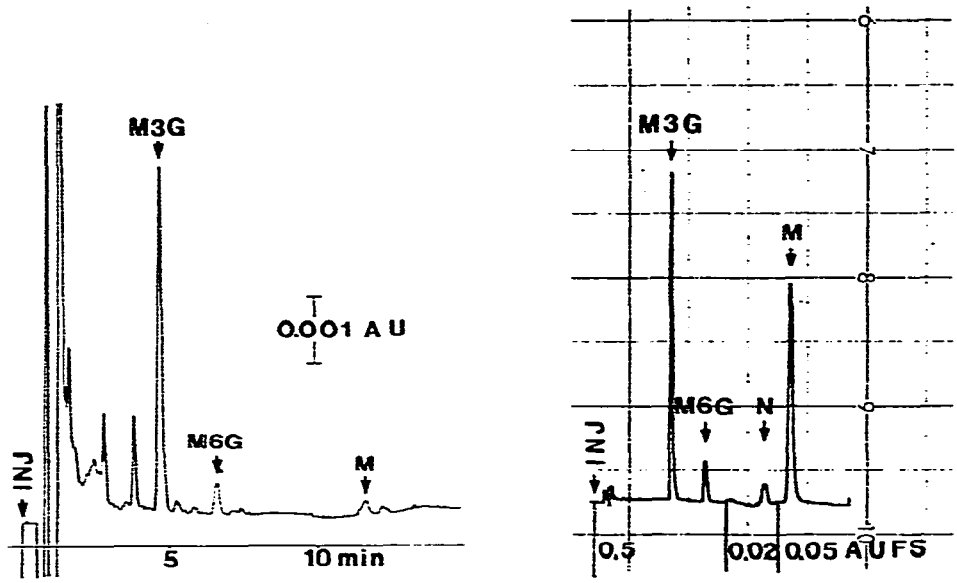

Fig. 2. Chromatogram of plasma from a morphine-treated cancer patient, with 244 ng/ml morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), and 9 ng/ml morphine (M).

Fig. 3. Chromatogram of urine from a morphine-treated cancer patient, with morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), normorphine (N) and morphine (M). Note the different sensitivities, indicated by absorbance units full scale (AUFS).

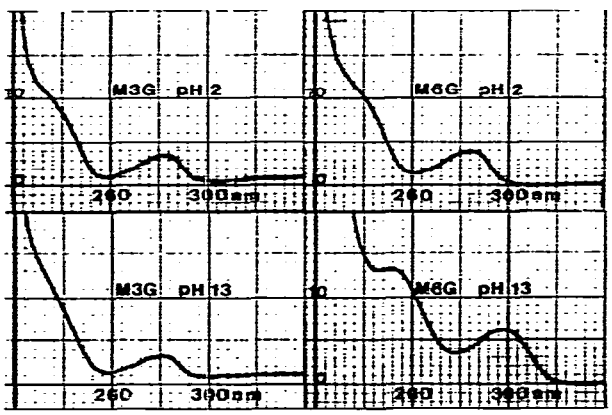

Fig. 4. UV spectra of morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) in acid and basic solution. A bathochromic shift is observed for morphine-6-glucuronide.

In chromatograms from human plasma and urine, a peak with an area of 5—20% of that of morphine-3-glucuronide was consistently found (see Figs. 2 and 3). This peak was tentatively identified as morphine-6-glucuronide in the following manner:

(A) A urine sample with high concentration of this substance was injected on the chromatograph, and the peak eluate was collected. The UV-absorbance curve of this fraction was similar to that of morphine-3-glucuronide. After

alkalinization it showed the bathochromic shift typical of a free phenolic group (Fig. 4) [3].

(B) After treatment of the collected fraction with 1 *M* hydrochloric acid for 1 h at 100°C, the peak area of the assumed morphine-6-glucuronide decreased by 20%. Concomitantly, a morphine peak appeared in the chromatogram. When the morphine-3-glucuronide fraction was treated in the same way, 87% of this metabolite was hydrolysed to morphine.

(C) To a diluted urine sample, containing about 80 μg/ml morphine-3-glucuronide and about 15 μg/ml morphine-6-glucuronide (assuming the same molar absorbance as for morphine-3-glucuronide) in a 0.1 *M* acetate buffer pH 5.5, 10% β-glucuronidase—arylsulphatase from *Helix pomatia* (Boehringer, Mannheim, G.F.R.) was added. The mixture was incubated for 24 h at 37°C. The morphine-3-glucuronide peak disappeared completely, and the peak area of morphine-6-glucuronide decreased by 74%. The morphine peak increased accordingly.

(D) When human liver microsomes (protein concentrations 4.5 mg/ml incubate) were incubated with 0.14 m*M* morphine and 13.5 m*M* uridine diphosphoglucuronic acid in a Tris—HCl buffer pH 8.7 [7], morphine-3-glucuronide and the assumed morphine-6-glucuronide were biosynthesized in about the same proportions as found in plasma.

Our method has been used in studies on morphine kinetics in cancer patients. No interfering peaks from concomitant drug therapy have been observed. However, in smoking patients, a tailing peak which precedes the tentative morphine-6-glucuronide peak might cause problems in the quantitation of

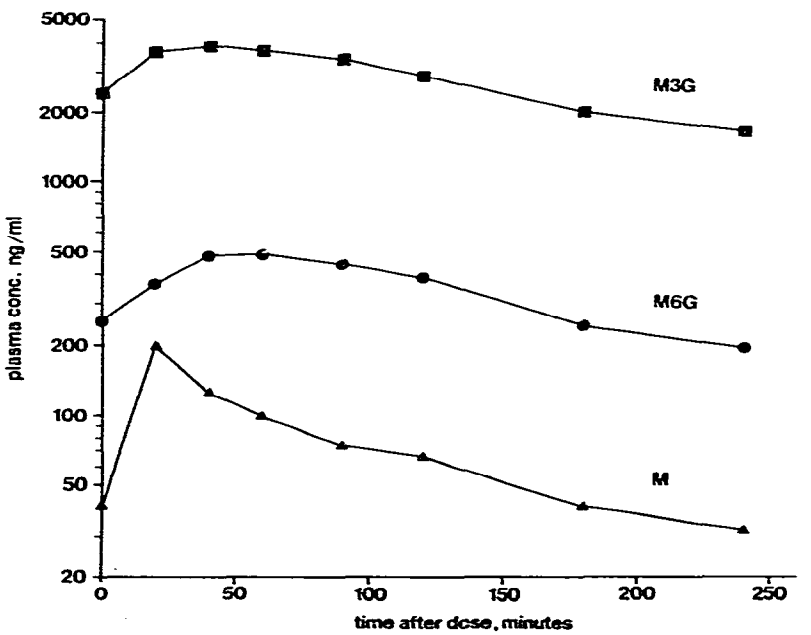

Fig. 5. Concentrations of morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G) and morphine (M) in plasma from a cancer patient treated with 100 mg of morphine hydrochloride orally at a regular 4-h dose interval.

low concentrations of this metabolite. Fig. 5 shows the plasma concentration of morphine and its glucuronide metabolites in a representative patient.

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