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Note

Method for determination of morphinone in urine and bile of guinea pig by high-performance liquid chromatography

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In our preliminary study, the urinary excretion of morphinone as a new metabolite of morphine in guinea pigs has been confirmed by isolations of the metabolite as 8-(2-hydroxyethylthio)dihydromorphinone (morphinone–2-mercaptoethanol adduct) because of its labile and decomposable nature [1]. We also reported that morphinone was about nine times more toxic than morphine in mice and bound irreversibly to tissue protein and opiate receptor [2–4]. Recently, NAD(P)-linked morphine 6-dehydrogenase, responsible for forming morphinone from morphine, has been purified from guinea pig liver and characterized extensively [5]. These findings strongly suggested that morphinone is the toxic factor of morphine and has a role in the development of tolerance. In order to clarify these possibilities, we sought to establish a method for the quantitation of morphinone in biological samples.

In this paper, we describe the separation of morphinone as its 2-mercaptoethanol adduct from morphine and other potential metabolites of morphine, such as normorphine, dihydromorphine and dihydromorphinone, by high-performance liquid chromatography (HPLC) using an octadecylsilane (ODS) column with residual silanol groups, and a simple and efficient extraction from urine and bile using a Sep-Pak C_{18} cartridge. This method is applicable to the determination of not only morphinone but also morphine and its unconjugated metabolites in the urine and bile of guinea pigs.

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EXPERIMENTAL

Materials

Morphinone, dihydromorphine and dihydromorphinone were synthesized by the method of Rapoport and co-workers [6, 7]. Normorphine was synthesized by the method of Braun [8]. These compounds were converted into the form of water-soluble hydrochlorides. Morphinone–2-mercaptoethanol adduct was synthesized as described previously [5]. Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). Acetonitrile was of HPLC grade. All reagents were of the highest grade available. All glassware used was siliconized with Aquasil (Pierce, Rockford, IL, U.S.A.). Before use, the Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were washed with 5 ml of methanol, 10 ml of water and 3 ml of 2 m*M* sodium phosphate buffer (pH 7.4).

Chromatographic conditions

The chromatographic equipment consisted of a Model 510 pump, a Model U6K injector, a Model 440 absorbance detector and an Extended Wavelength Module (Waters Assoc.). A 150×6 mm I.D. YMC AL-312 (ODS) packed column (5-μm particles; Yamamura Chemical Labs., Kyoto, Japan) was used, with a 10×4 mm I.D. precolumn containing LiChrosorb RP-18 (10-μm particles; E. Merck, Darmstadt, F.R.G.). The separation was carried isocratically with 10 m*M* sodium phosphate buffer (pH 6.8)–acetonitrile (3:2, v/v) at ambient temperature. The flow-rate was 2 ml/min and the peaks were monitored at 214 nm (0.02 a.u.f.s.). The peak areas were determined by a Shimadzu Chromatopac C-R1B integrator (Kyoto, Japan).

Calibration curve

Morphinone–2-mercaptoethanol adduct, morphine, normorphine, dihydromorphine or dihydromorphinone was dissolved in water to provide a standard solution with a concentration of 10 μg/ml as free base. The calibration curve was obtained by injecting the various volumes (2–40 μl) of each standard solution and conducted by plotting the peak area against the injected amount.

Recovery

Urine. A 4-ml volume of urine was mixed with 1 ml of 2 *M* hydrochloric acid and spiked with 100 μmol of 2-mercaptoethanol. This solution was adjusted to pH 7 with 4 *M* sodium hydroxide, mixed with 5 ml of 0.2 *M* sodium phosphate buffer (pH 7.4) and then allowed to stand for 30 min at room temperature. After centrifugation at 3000 *g* for 10 min, the supernatant was made up to 10.0 ml with 0.2 *M* sodium phosphate buffer (pH 7.4) and then passed through a 0.45-μm membrane filter (Toyo Roshi, Tokyo, Japan). A 1-ml volume of the filtrate was poured onto a Sep-Pak C_{18} cartridge, washed with 20 ml of water and eluted with 1.5 ml of methanol. The methanol eluate was used for estimating the background in HPLC. In recovery experiments, 100 μg of morphinone, morphine, normorphine, dihydromorphine or dihydromorphinone were added to 5 ml of urine containing hydrochloric acid, and then subjected to the procedure described above.

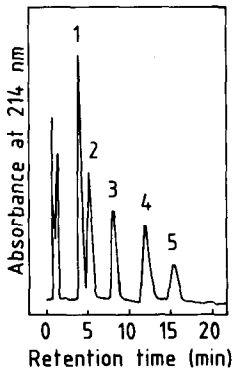

Fig. 1. Separation of a mixture of normorphine (1), morphinone–2-mercaptoethanol adduct (2), morphine (3), dihydromorphine (4) and dihydromorphinone (5) on a YMC AL-312 (ODS) column. Mobile phase, 10 m*M* sodium phosphate buffer (pH 6.8)–acetonitrile (3:2, v/v); flow-rate, 2 ml/min; 100 ng of each compound were injected.

Triplicate samples were determined by the peak area compared with that obtained from the calibration curve.

Bile. A 1-ml volume of bile containing 50 μmol of 2-mercaptoethanol with or without morphinone, morphine, normorphine, dihydromorphine or dihydromorphinone was mixed with 4 ml of 0.2 *M* sodium phosphate buffer (pH 7.4), allowed to stand for 30 min and passed through a membrane filter. Then 4 ml of the filtrate were treated as described for the urine. The methanol eluates obtained were used for the estimation of the background and the determination of recovery from bile.

RESULTS AND DISCUSSION

Considering the instability of morphinone, quantitation of morphinone as its 2-mercaptoethanol adduct is better than that of morphinone itself. Morphinone–2-mercaptoethanol adduct was quantitatively and easily formed from morphinone in the presence of a large amount of 2-mercaptoethanol in aqueous solution at pH above 7. Accordingly, we first attempted the separation of morphinone–2-mercaptoethanol adduct from morphine and its unconjugated metabolites by HPLC. A typical chromatogram on an ODS column with residual silanol groups (AL type) is shown in Fig. 1. All compounds examined were well resolved from each other with symmetrical peaks, except that the dihydromorphine peak showed a little tailing. The peak of free morphinone appeared at 8.4 min and was inadequately resolved from that of morphine. This result indicates that it is better to use morphinone–2-mercaptoethanol adduct than morphinone itself in the analysis.

On an AL-type column, the separation and the retention times of these compounds were markedly affected by the pH of the mobile phase, and satisfactory separation was achieved at pH values above 6.5. With increasing pH or decreasing ionic strength of the mobile phase, the retention times of these compounds

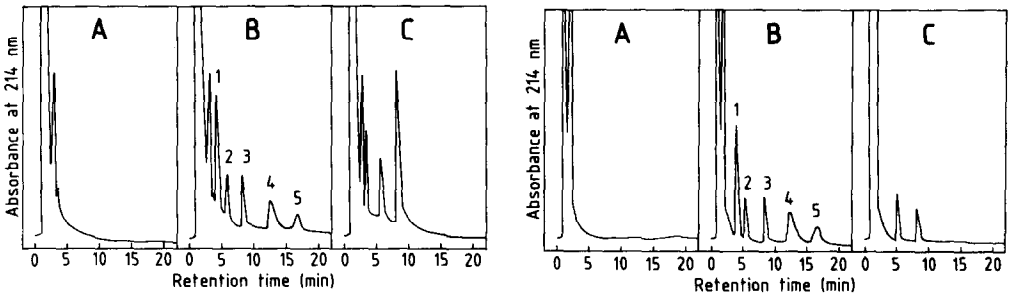

Fig. 2. Chromatograms of blank urine extract (A), urine extract spiked with 10 μg each of normorphine, morphinone-2-mercaptoethanol adduct, morphine, dihydromorphine and dihydromorphinone (B), and urine sample extract obtained from a guinea pig given morphine (C). Peaks: 1 = normorphine; 2 = morphinone-2-mercaptoethanol adduct; 3 = morphine; 4 = dihydromorphine; 5 = dihydromorphinone. The urine sample was taken 24 h after subcutaneous injection of morphine (25 mg/kg). The chromatographic conditions are the same as in Fig. 1.

Fig. 3. Chromatograms of blank bile extract (A), bile extract spiked with 10 μg each of normorphine, morphinone-2-mercaptoethanol adduct, morphine, dihydromorphine and dihydromorphinone (B), and bile sample extract obtained from a guinea pig given morphine (C). Peaks: 1 = normorphine; 2 = morphinone-2-mercaptoethanol adduct; 3 = morphine; 4 = dihydromorphine; 5 = dihydromorphinone. The bile sample was taken 60 min after subcutaneous injection of morphine (25 mg/kg). The chromatographic conditions are the same as in Fig. 1.

increased. On the other hand, when an ODS column without residual silanol groups (A type) was employed, all the compounds eluted within 2 min with the same mobile phase as shown in Fig. 1. Moreover, their retention times were not appreciably changed by varying the pH of the mobile phase in the range 4–8. From these results, it appears that the separation on the ODS column is probably due to a dual retention mechanism involving both the ODS groups and the residual silanol groups, which could interact with the secondary or tertiary amine group of the compounds.

The linear response between the peak area and the injected amount of morphinone-2-mercaptoethanol adduct was obtained over the range 20–400 ng, and a linear regression was expressed as the equation: $y = 0.01x - 0.05$ (where y is the peak area in integrated count $\times 10^{-4}$ and x is the injected amount in ng) with a coefficient of correlation of 0.999. The lower limit of detection was 10 ng. Calibration curves for morphine, normorphine, dihydromorphine and dihydromorphinone were also linear over the range 20–400 ng, and the lower limits of detection were 10 ng for morphine and normorphine and 20 ng for dihydromorphine and dihydromorphinone.

Next, we examined the background of the extracts from urine and bile in HPLC and the recoveries of morphinone, morphine, normorphine, dihydromorphine and dihydromorphinone from urine and bile. In these experiments, the urine and bile containing hydrochloric acid and/or 2-mercaptoethanol were used in order to prevent the decomposition of morphinone and derivatize morphinone to its 2-mercaptoethanol adduct. Figs. 2A and 3A show the chromatograms of the methanol eluates of the blank urine and bile from the Sep-Pak C_{18} cartridge. Neither

TABLE I

RECOVERIES AND REPRODUCIBILITIES OF MORPHINONE, MORPHINE, NORMORPHINE, DIHYDROMORPHINE AND DIHYDROMORPHINONE FROM THE URINE AND BILE BY THE EXTRACTION WITH SEP-PAK C_{18} CARTRIDGE

Compound	Urine		Bile	
	Recovery★ (%)	C.V. (%)	Recovery★ (%)	C.V. (%)
Morphinone	83.6	4.4	84.0	2.3
Morphine	91.4	2.3	101.4	2.3
Normorphine	92.7	1.5	97.9	4.6
Dihydromorphine	93.2	1.6	95.4	1.0
Dihydromorphinone	76.2	4.1	77.3	3.9

★Mean of three determinations.

eluate afforded any peaks that interfered with those of the morphinone–2-mercaptoethanol adduct, morphine and other metabolites. The methanol eluates spiked with these compounds provided a baseline separation between these compounds and the backgrounds in both cases (Figs. 2B and 3B). Typical chromatograms of urine and bile sample extracts obtained from guinea pigs given morphine are shown in Figs. 2C and 3C. Peaks due to the morphinone–2-mercaptoethanol adduct and morphine were observed, but normorphine, dihydromorphine and dihydromorphinone were absent from both chromatograms. Other than the unconjugated metabolites described above, guinea pigs given morphine excrete morphine-3-glucuronide and morphine-6-glucuronide in the urine and bile. However, these polar metabolites were eluted within 1.5 min under the present conditions and gave no interfering peaks.

Table I shows the mean recoveries of morphinone, morphine, normorphine, dihydromorphine and dihydromorphinone from the urine and bile and the coefficient of variation (C.V.) for analysis in three experiments. Morphinone was quantitated as morphinone–2-mercaptoethanol adduct in this study. Complete conversion of morphinone–2-mercaptoethanol adduct in the urine and bile was ascertained because the peak corresponding to morphinone was absent from the chromatogram. Despite its instability, more than 80% of morphinone was recovered from both the urine and bile, and the good reproducibility was indicated by a C.V. of 4.4% for urine analysis and 2.3% for bile analysis. When urine and bile without hydrochloric acid and/or 2-mercaptoethanol were used in the experiments, the recovery of morphinone apparently decreased in both cases. Following prolonged standing before extraction, the recovery decreased further. Accordingly, for accurate determination of morphinone in urine and bile, it is necessary to collect these samples in the reservoir containing hydrochloric acid and/or 2-mercaptoethanol.

The present method developed for the determination of morphinone also provided good recoveries for morphine, normorphine and dihydromorphine from urine and bile, with good reproducibility (Table I). Recovery of dihydromorphinone was somewhat lower than those of the other compounds. Cone et al. [9] reported

the recoveries of these compounds (except morphinone) from urine by a gas chromatographic–mass fragmentographic method, which involved solvent extraction and derivatization to volatile compounds prior to analysis. In comparison with their method, the present method is simple and rapid and gives a markedly improved recovery of normorphine.

In conclusion, we have developed a reliable method for the quantitative determination of morphinone in urine and bile. It involves conversion into the stable 2-mercaptoethanol derivative and extraction and clean-up with a Sep-Pak C_{18} cartridge prior to isocratic HPLC on an ODS column with residual silanol groups. With the present method it is possible to determine accurately morphinone concentrations as low as 0.5 μg/ml in urine and 0.2 μg/ml in bile. This method also enables a simultaneous determination of morphine and its unconjugated metabolites, and it is applicable to studies of the in vivo and in vitro metabolism of morphine.

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