(18) H. Brem, A. B. Stein, and H. S. Rosenkranz, *Cancer Res.*, **34**, 2576(1974).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 18, 1974, from the *Departments of Microbiology and Pediatrics, College of Physicians and Surgeons, Columbia University, New York, NY 10032*

Accepted for publication November 29, 1974.

Supported by the Division of Cancer Cause and Prevention, National Cancer Institute, under Contract NO1 CP-33395 and the George A. Carden, Jr. Special Fund for Cancer Research.

* Vivian Allan Fellow in Pediatrics.

‡ Research Career Development Awardee of the National Institute of General Medical Sciences, 5 K3-GM 29,024.

ˣ To whom inquiries should be directed.

GLC Determination of Heroin and Its Metabolites in Human Urine

S. Y. YEH ˣ and R. L. McQUINN

Abstract □ Heroin and its metabolites, 6-monoacetylmorphine, morphine, and normorphine, were determined in human urine with a GLC procedure. Heroin was extracted with chloroform at pH 4.5 and chromatographed at a temperature programmed from 200–250° by 8°/min. 6-Monoacetylmorphine and morphine were extracted with ethylene dichloride containing 30% isopropanol at pH 8.5, and normorphine was extracted at pH 10.4 with the same solvent. The extract was derivatized with trimethylsilylimidazole and chromatographed at 230° for the determination of 6-monoacetylmorphine and morphine and at 220° for normorphine and morphine.

Keyphrases □ Heroin and metabolites—GLC analysis in human urine □ Morphine, normorphine, and 6-monoacetylmorphine (heroin metabolites)—GLC analysis in human urine □ GLC—analysis, heroin and metabolites in human urine

Previous studies on laboratory animals *in vitro* and *in vivo* have indicated that heroin (3,6-diacetylmorphine) is rapidly deacetylated to 6-monoacetylmorphine and then to morphine (1–7). The major portion of a given dose of heroin can be accounted for in the urine as free morphine and morphine conjugate (8–13). Heroin, 6-monoacetylmorphine, and morphine have previously been estimated in biological materials with a methyl orange dye procedure, a Folin–Ciocalteu phenolic reagent (3), radioactive tracers (5), paper chromatography (7, 14), and GLC (12). This article presents an improved GLC procedure for the estimation of heroin and its metabolites, 6-monoacetylmorphine, morphine, and normorphine.

EXPERIMENTAL

Materials—Heroin hydrochloride[1] (containing about 5% 6-monoacetylmorphine as an impurity, as determined with the GLC procedure described here), normorphine hydrochloride[1], and morphine sulfate[2] USP were obtained.

Gas Chromatograph—A gas chromatograph[3] equipped with dual flame-ionization detectors and a dual pen recorder[4] was used. A 0.9-m (3-ft) × 2-mm glass column (Column 1) was packed with 3% OV-17 coated on Gas Chrom Q (60–80 mesh) and conditioned at 270° by passing 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. A 1.5-m (5-ft) × 2-mm stainless steel column (Column 2) was packed with 3% SE-30 coated on Varaport (100–120 mesh). The temperatures of the injector and detector were set at 255 and 295°, respectively.

Determination of Heroin—A 5-ml aliquot of urine was placed in a 40-ml centrifuge tube, adjusted to about pH 4.5 with acetic acid, buffered with 1 ml of 1 *M* sodium acetate buffer at pH 4.5, salted with 1.6 g sodium chloride, shaken with 15 ml of chloroform in a shaker[5] at 280–300 oscillations/min for 10 min, and centrifuged at 2000 rpm for 10 min. After removal of the aqueous phase by aspiration, a 13-ml aliquot of the organic phase was transferred to a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen in a water bath at 60–70°.

The tube was rinsed with 1 ml of chloroform by mixing on a mixer[6] for about 10 sec, and the chloroform was evaporated to dryness. To the residue was added 50 μl of cholestane solution (0.05 mg/ml in ethyl acetate) as an internal standard, and 1 μl of the solution was injected onto Column 2. The temperature of the column was programmed from 200 to 250° at 8°/min. A standard curve was prepared by adding authentic heroin hydrochloride (0.25–10 μg) to 5 ml of drug-free cigarette smoker's urine and analyzing by the described procedure.

Determination of 6-Monoacetylmorphine and Morphine—A 5-ml aliquot of urine was placed in a 40-ml centrifuge tube, adjusted to pH 8.0–8.5 with sodium hydroxide solution, buffered with 1 ml of 1 *M* phosphate buffer at pH 8.5, salted with 1.6 g of sodium chloride, shaken with 15 ml of ethylene dichloride containing 30% isopropanol in a shaker[5] at 280–300 oscillations/min for 10 min, and centrifuged at 2000 rpm for 10 min. After removal of the aqueous phase by aspiration, a 13-ml aliquot of the organic phase was transferred to a 40-ml centrifuge tube.

The alkaloids in the organic phase were back-extracted into the aqueous phase with 5 ml of 0.1 *N* HCl by shaking in the shaker for 5 min and centrifuging for 5 min. The organic phase was removed by aspiration without removing any of the acid phase (leaving a small drop of the organic phase in the bottom of the tube). The acidic phase was adjusted to pH 8.0–8.5, buffered, salted with 1.0 g of sodium chloride, and extracted with the organic solvent as already described. After removal of the aqueous phase by aspiration, a 13-ml aliquot of the organic phase was transferred carefully (so as not to include any aqueous phase) to a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen in a water bath at 60–70°.

[1] Through the courtesy of Dr. Everette May, National Institutes of Health.

[2] Commercial product.

[3] Varian Aerograph, Series 2700.

[4] Model A-25, Varian.

[5] International bottle shaker, International Equipment Co., Boston, Mass.

[6] Vortex.

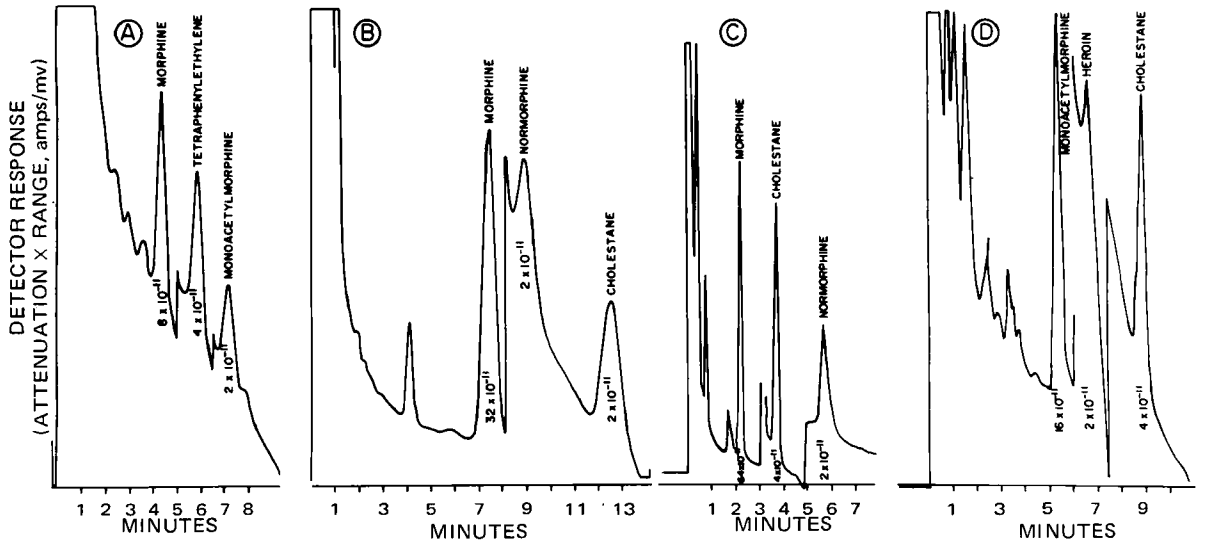

Figure 1—*Chromatograms of heroin, 6-monoacetylmorphine, morphine, and normorphine obtained from the extract of human urine after intravenous administration of 10 mg/70 kg of heroin hydrochloride.*

The tube was rinsed with 1 ml of methanol by mixing on a mixer[6] for about 10 sec and was then evaporated to dryness. The residue was used for GLC determination of 6-monoacetylmorphine and morphine after silanization with 25% trimethylsilylimidazole in pyridine[7]. Standard curves were prepared with five concentrations of 6-monoacetylmorphine and morphine added to 5 ml of drug-free cigarette smoker's urine and analyzed as already described.

Determination of Morphine and Normorphine—The procedure for extraction of normorphine and morphine was described previously (15). It was essentially the same as the procedure for extraction of 6-monoacetylmorphine and morphine, except that the urine samples were adjusted to about pH 10, buffered at pH 10.4 with 1 ml of 40% phosphate buffer (37% K_2HPO_4 and 3% K_3PO_4), and extracted with ethylene dichloride (glass distilled) containing 30% isopropanol by shaking for 30 min each time.

The residue of the urine extract was dissolved in 0.3 ml of methanol, 50 μl of internal standard solution was added, and the tube was mixed[6] for about 10 sec. Tetraphenylethylene (50 μg/ml in ethyl acetate) was used for the determination of 6-monoacetylmorphine and morphine, and cholestane solution (50 μg/ml in ethyl acetate) was used for the determination of normorphine. The solution was transferred to an acylation tube[8], and the centrifuge tube was rinsed with 0.1 ml of methanol and transferred to the acylation tube. The mixture was evaporated to dryness, and the residue was either silanized or acetylated.

Acetylation was accomplished by heating the residue with 0.2 ml of acetic anhydride and 0.1 ml of pyridine in a sealed acylation tube at 60–70° for 0.5 hr. The excess acetic anhydride was removed by evaporation and the residue was dissolved in 50 μl of ethyl acetate; 1 μl of the solution was injected onto Column 2. Silanized derivatives were prepared by mixing the contents of the tube with 50 μl of 25% trimethylsilylimidazole in pyridine and heating the mixture in an oil bath at 90–95° for 1 hr. One microliter of the solution was injected onto Column 1.

The ratios of the peak heights of heroin, 6-monoacetylmorphine, morphine, and normorphine to the peak height of internal standard were calculated. A linear relationship was found by plotting the peak height ratios *versus* concentrations. Heroin, 6-monoacetylmorphine, morphine, and normorphine concentrations in the urine sample were determined from the standard curves. The amount of injected heroin was converted to equivalent amounts of 6-monoacetylmorphine and morphine for calculation of the percentage of excretion.

Estimation of Free and Conjugated Morphine and Normorphine—Total morphine and normorphine in the urine were estimated, according to the precedure of Yeh and Woods (16), by autoclaving the specimen in a final concentration of 2.2 *N* HCl (20% by volume of concentrated hydrochloric acid) in a steam-jacketed autoclave at 15 lb of pressure and 115° for 30 min. After cooling, the sample was adjusted to about pH 10 and then extracted for GLC by the described procedure. Free morphine and normorphine were estimated from the unhydrolyzed specimens. Conjugated morphine was calculated as the difference between the total morphine and the free drug of morphine and 6-monoacetylmorphine.

RESULTS AND DISCUSSION

The chromatograms of heroin and its metabolites (6-monoacetylmorphine, morphine, and normorphine) extracted from the first urine samples of subjects after intravenous administration of 10 mg of heroin hydrochloride/70 kg are shown in Fig. 1. Normorphine, either in free or conjugated form, would be expected to be a metabolite of heroin since free and conjugated normorphine have been found in the urine of morphine-dependent subjects (15). The chromatograms of free 6-monoacetylmorphine and free morphine Fig. 1(A) were obtained from a silanized extract and chromatographed on Column 1 at a temperature of 230°. The retention times of silylmorphine, tetraphenylethylene, and silyl-6-monoacetylmorphine were 4.3, 5.8, and 7.1, respectively.

Chromatograms B and C show total morphine and total normorphine extracted from acid-hydrolyzed urine at pH 10.4. Chromatogram B was obtained from the extract that was silanized and chromatographed on Column 1 at 220°. The retention times of silylmorphine, silylnormorphine, and cholestane under these conditions were 7.4, 8.8, and 12.5 min, respectively. Chromatogram C was obtained from the extract that was acetylated and chromatographed on Column 2 at 250°. The retention times of diacetylmorphine, cholestane, and triacetylnormorphine were 2.3, 3.7, and 5.7 min, respectively.

Chromatogram D shows 6-monoacetylmorphine and heroin obtained from the extract chromatographed on Column 2, temperature programmed from 200 to 250° at 8°/min. The retention time of heroin and its metabolites changed slightly from column to column but the basic pattern remained the same. Free normorphine concentration was too low to be detectable in a 5-ml urine aliquot but was detected from larger samples extracted with XAD-2 resin[9].

The sensitivity of the procedure for determination of heroin was

[7] Tri-Sil-Z, Pierce Chemical Co., Rockford, Ill.
[8] Regis Chemical Co., Chicago, Ill.
[9] Amberlite XAD-2 resin, Rohm & Haas Chemical Co., Philadelphia, Pa.

0.2 μg/5 mg of urine. The extraction efficiency of the procedure could not be established, because the volatility of heroin hydrochloride appears higher than that of heroin (presumably heroin acetate) extracted with the solvent. The peak height of heroin hydrochloride (2–10 μg) was less than that of the same amount of heroin after extraction when chromatographed on Column 1. Column 2 seemed more sensitive than Column 1 when extracted heroin was chromatographed, although heroin hydrochloride did not emerge from Column 2.

The extract obtained from a single extraction contained many impurities, but these did not interfere with the detection or measurement of the heroin peak. Attempts to clean the extract by extraction of heroin into the aqueous phase with 0.1 *N* HCl and then back into the organic phase with chloroform at pH 4.5 were unsuccessful. 6-Monoacetylmorphine was also extracted with chloroform from the urine sample buffered at pH 4.5.

6-Monoacetylmorphine is quite stable under the conditions for extraction and silanization, as evidenced by the fact that a morphine peak was not observed in the chromatogram when authentic 6-monoacetylmorphine alone was added to the urine. Attention must be paid to the adjustment of the pH of the sample. Oberst (10) reported that 88% of heroin was hydrolyzed at 26° in 0.5 *M* sodium carbonate solution in 10 min. The biological sample obtained after administration of heroin should be frozen and analyzed as soon as possible. 6-Monoacetylmorphine was detected in 10 out of 10 frozen human urine samples and only in three out of 10 refrigerated urine samples after heroin administration.

The overall recoveries for 6-monoacetylmorphine and morphine in concentrations of 2–15 μg/5 ml of urine were 68–70 and 60–62%, respectively. The sensitivity of this procedure for the determination of 6-monoacetylmorphine hydrochloride and morphine sulfate was 0.25 μg/5 ml of urine. Morphine can be extracted from the sample buffered to pH 8.5 or 10.4 and determined simultaneously with either 6-monoacetylmorphine or normorphine.

The optimal conditions of reaction time and temperatures for derivatization of morphine and normorphine were studied with two silanizing reagents, bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane and 25% trimethylsilylimidazole in pyridine. A clean chromatogram was obtained from the extract of a drug-free urine sample when trimethylsilylimidazole was used. The results from silanization of 100 μg of morphine sulfate and normorphine hydrochloride with 50 μl of 25% trimethylsilylimidazole in pyridine heated at 90–95° for 15, 30, and 60 min (six samples for each time period) were not significantly different. However, the chromatogram of drug-free urine extracts heated for 15 and 30 min with the silanizing reagent showed a small peak interfering with morphine (0.2 min after morphine peak). The peak height ratios for morphine were not significantly different when the extract was silanized at 60–70 or 90–95°, but the peak height ratios for normorphine were smaller at the lower temperature. Therefore, reaction at 90–95° for 60 min was used in the final procedure.

The sensitivity of silanized and acetylated morphine is comparable but the sensitivity for determination of silanized normorphine is somewhat greater than that for acetylated normorphine.

Cholestane interferes with the retention time of 6-monoacetylmorphine, so it is not suitable for use as an internal standard for 6-monoacetylmorphine determination. Tetraphenylethylene was used as an internal standard for the determination of both 6-monoacetylmorphine and morphine.

This procedure is currently being used in this laboratory for studying the urinary excretion of heroin and its metabolites following intravenous administration of heroin in humans.

REFERENCES

(1) N. B. Eddy and H. A. Howes, *J. Pharmacol. Exp. Ther.*, **53**, 430(1935).

(2) J. W. Kemp and E. L. Way, *Fed. Proc.*, **18**, 409(1959).

(3) E. L. Way, J. M. Young, and J. W. Kemp, *Bull. Narcotics*, 17(1), 25(1965).

(4) E. L. Way, J. W. Kemp, J. M. Young, and D. R. Grassatti, *J. Pharmacol. Exp. Ther.*, **129**, 144(1960).

(5) E. L. Way and T. K. Adler, *Bull. WHO*, **26**, 51(1962).

(6) G. L. Cohn, J. A. Cramer, and H. D. Kleber, *Proc. Soc. Exp. Biol. Med.*, **144**, 351(1973).

(7) G. R. Nakamura and T. Ukita, *J. Pharm. Sci.*, **56**, 294(1967).

(8) T.-M. Shen, *J. Chin. Pharm. Ass.*, **1**, 124(1936).

(9) F. W. Oberst, *J. Pharmacol. Exp. Ther.*, **73**, 401(1941).

(10) *Ibid.*, **79**, 266(1943).

(11) B. P. N. Mo and E. L. Way, *J. Pharmacol. Exp. Ther.*, **154**, 142(1966).

(12) H. W. Elliott, K. D. Parker, J. A. Wright, and N. Nomof, *Clin. Pharmacol. Ther.*, **12**, 806(1972).

(13) C. W. Gorodetzky, C. R. Angel, D. J. Beach, D. H. Catlin, and S. Y. Yeh, *ibid.*, **15**, 461(1974).

(14) A. Stolman and C. P. Stewart, *Analyst*, **74**, 536(1949).

(15) S. Y. Yeh, *Fed. Proc.*, **32**, 763(1973); *J. Pharmacol. Exp. Ther.*, **192**, 201(1975).

(16) S. Y. Yeh and L. A. Woods, *J. Pharm. Sci.*, **59**, 380(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 17, 1974, from the *Division of Research, Addiction Research Center, National Institute on Drug Abuse, U.S. Department of Health, Education, and Welfare, Lexington, KY 40511*

Accepted for publication December 5, 1974.

[x] To whom inquiries should be directed.