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A GAS–LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NALTREXONE AND BETA-NALTREXOL IN HUMAN URINE

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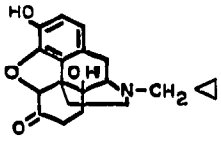
SUMMARY

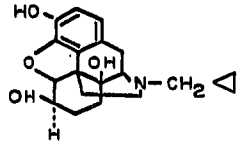
A rapid quantitative method was developed to assay in urine naltrexone and its major urinary metabolite, beta-naltrexol. Following solvent extraction and elimination of interfering materials, the weakly basic drug, its metabolite and the internal standard (etorphine) were silylated and analyzed by gas–liquid chromatography. As little as 0.02 μg/ml of naltrexone and beta-naltrexol was detectable using a hydrogen flame ionization detector. Twenty-four-hour urine samples were analyzed from three subjects taking 180 mg naltrexone daily. The major urinary excretion product was beta-naltrexol which accounted for 48.6% of the administered dose. Only 5% of the administered dose excreted in the urine was naltrexone. Beta-naltrexol was excreted 70% as free drug and 30% conjugated while naltrexone was 90% conjugated.

INTRODUCTION

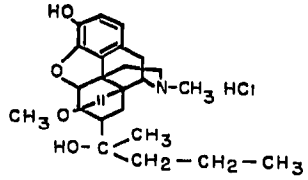
Naltrexone or N-cyclopropylmethylnoroxymorphone (Fig. 1) is a potent,



NALTREXONE



BETA-NALTREXOL



ETORPHINE

Fig. 1. Structural formulae of naltrexone, beta-naltrexol and etorphine (internal standard).

reasonably pure narcotic antagonist used as a therapeutic agent for the treatment of opiate dependence. It was synthesized by Blumberg *et al.*[1] in 1965. Recent clinical studies indicate that naltrexone is orally effective at considerably lower doses than naloxone and appears to have fewer undesirable side effects than either naloxone or cyclazocine[2,3] when used to antagonize the agonistic effects of opiates. Naltrexone is biotransformed by a keto reduction at the C-6 beta position producing primarily beta-naltrexol in man (Fig. 1). This major human urinary metabolite was isolated by Cone[4] and the ascribed beta configuration subsequently confirmed by Chatterjie *et al.*[5]. Urinary excretion data in man and a gas–liquid chromatographic (GLC) method for the quantitation of naltrexone and beta-naltrexol has been described by Cone *et al.*[6].

In this report a clinically applicable, rapid method for the quantitation of both naltrexone and beta-naltrexol in urine utilizing GLC is described.

MATERIALS AND METHODS

Chemicals and reagents

Naltrexone (N-cyclopropylmethylnoroxymorphone, EN 1639A) was kindly provided by Dr. H. Blumberg of Endo Laboratories, Garden City, N.Y., U.S.A. Beta-naltrexol (N-cyclopropylmethyl-7,8-dihydro-14-hydroxynorisomorphine) was a kind gift from Dr. Cone of NIDA, Addiction Research Center, Lexington, Ky., U.S.A. Etorphine (7,8-dihydro-7-[1(R)-hydroxy-1-methylbutyl]-O^6-methyl-6,14-endo-ethenomorphine) was purchased from American Cyanamid, Princeton, N.J., U.S.A. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Regis, Morton Grove, Ill., U.S.A. All organic solvents used in the extraction procedure were glass distilled, G.C. grade, obtained from Burdick and Jackson Labs., Muskeegan, Mich., U.S.A. A reagent kit for the determination of creatinine was purchased from Pierce, Rockford, Ill., U.S.A.

Stock solutions

Standard solutions of naltrexone (20 μg/ml), beta-naltrexol (300 μg/ml) and etorphine (20 and 200 μg/ml) were prepared. The buffer was 0.2 *M* Delory-King (D/K) carbonate–bicarbonate buffer at pH 8.5 (ref. 7).

Extraction procedure

To assay for naltrexone 1–2 ml of urine was needed, while for the determination of beta-naltrexol 0.1–0.3 ml urine was sufficient. Etorphine, the internal standard, was added directly to the urine samples: 2 μg for the naltrexone assay and 20 μg for the beta-naltrexol assay.

The urines were buffered to pH 8.5 using 0.5 ml of D/K buffer and the aqueous solution extracted with 7.0 ml of chloroform. The chloroform extracts of the weak organic bases were extracted into 5.0 ml of 0.5 *N* HCl. The pH of the aqueous layer was adjusted to 8.5 and re-extracted into 7.0 ml chloroform. The extractions at each step consisted of 10 min shaking in an Eberbach shaker (Eberbach, Ann Arbor, Mich., U.S.A.) and 5 min centrifugation in an International Equipment (Needham Hts., Mass., U.S.A.) centrifuge Model K at 1500 rpm. The chloroform extracts were evaporated to dryness in a rotary evaporator under vacuum at 50° (Evapomix, Buchler, Fort Lee, N.J., U.S.A.).

Silylation procedure

20–30 μl BSTFA were added to siliconized test tubes (15 × 75 mm) used for the evaporation of the final chloroform extract (last step of the extraction method). The tubes were sealed with rubber caps, flushed with dry nitrogen, mixed on a Vortex mixer for 5 sec and the reaction allowed to proceed in a dry heating block containing sea sand at 60° for 15 min. After 3 min incubation the tubes were re-mixed on the Vortex mixer for 5 sec to assure that the free bases dissolved in the warm BSTFA and then replaced in the heating block for the remainder of the incubation period. Following the reaction 1–2 μl of BSTFA containing silylated bases was injected into the gas chromatograph.

Gas–liquid chromatographic conditions

The GLC analysis was performed on a Perkin Elmer 900 gas chromatograph, equipped with a hydrogen flame ionization detector. The column was a 6 ft. × 2 mm I.D. glass spiral. The packing consisted of 3% OV-17 on Gas-Chrom Q, 80–100 mesh. The temperature of the detector and flash heater was 285°. The carrier gas was helium with a flow-rate of 60 ml/min. The flow-rates of hydrogen and air were 30 ml/min and 300 ml/min, respectively. The column oven temperature was 270°.

Calibration curves and quantitation

For the quantitation of naltrexone standard curves were prepared by adding 0.5, 1.0, 1.5 and 2.0 μg of naltrexone and 2.0 μg of etorphine as the internal standard to each 2.0 ml of blank urine samples. The standard curves for beta-naltrexol were established by adding 3, 6, 9 and 12 μg beta-naltrexol and 20 μg etorphine to each 0.2 ml of blank urine samples. The samples for both standard curves were extracted according to the method described above, derivatized with BSTFA and chromatographed. The peak-height ratios were plotted against the respective concentration of standards and the slope factor was calculated. The unknown-sample peak-height ratio was multiplied by the respective slope factor to determine the quantity of naltrexone or beta-naltrexol in the unknown samples.

Isolation of beta-naltrexol from human urine

A large aliquot (500 ml) of the urine from a subject taking 160 mg naltrexone daily was hydrolyzed using Glusulase (Endo Labs., Garden City, N.Y., U.S.A.) and extracted according to the method described above. Following evaporation the residue was dissolved in absolute ethanol and applied on preparative thin-layer chromatographic plates (silica gel ITLC plates from Gelman). The chromatograms were developed with a solvent system of *n*-hexane (60 ml), ethylacetate (40 ml) and conc. ammonia (0.2 ml). The spots were visualized using iodoplatinate spray reagent.

Glusulase and acid hydrolysis

The concentration of urinary naltrexone and beta-naltrexol was determined before (free) and after hydrolysis (total). Subtraction of the free base from the total base yields the conjugated base. Glusulase hydrolysis was performed according to the method of Levy and Yamada[8]. Acid hydrolysis was also performed by adding equal volumes of conc. hydrochloric acid and urine, mixing and boiling for 1–1.5 min. The samples were cooled, neutralized with sodium hydroxide and the pH adjusted to 8.5 with 1.0 ml D/K buffer and extracted as described previously.

Creatinine determination

Urinary creatinine levels were determined, using a kit purchased from Pierce, by the method of Slot[9].

RESULTS AND DISCUSSION

A solvent extraction and sample purification system has been developed for the quantitation of naltrexone and beta-naltrexol in the urine of patients taking naltrexone. All three compounds are structurally similar weak bases (Fig. 1), thus their partition properties between aqueous and organic solvents are thought to be similar. Etorphine was chosen as the internal standard because: (1) it follows the unknown compound through the extraction and purification steps; (2) it derivatizes predictably with BSTFA yielding a single symmetrical major peak; (3) the retention time of etorphine is longer than both naltrexone and beta-naltrexol emerging from the column at a zone where no interfering peaks are present both in blank urine and in the extracts of urine from patients who were taking naltrexone. The pH-dependent transfer of the three bases between aqueous and organic solvents eliminated contaminants and the chromatograms were reasonably free of non-specific or interfering peaks (Fig. 3). Using this method the recoveries of 2 μg naltrexone, beta-naltrexol and etorphine ($\pm$ standard error) from urine were 96.8 $\pm$ 0.99%, 82.2 $\pm$ 1.2%, and 86.6 $\pm$ 0.91%, respectively.

Both naltrexone and beta-naltrexol yielded relatively insensitive and tailing peaks when chromatographed on columns packed with various stationary liquid phases, *e.g.*, 3% SE-30, 3% OV-17, 3% XE-60 and 3% QF-1. To increase the sensitivity and improve the peak symmetry the extracts were derivatized with various silylating agents. The most consistent conversion resulting in a single major product was obtained with BSTFA. The derivatization was not reproducible under all conditions, thus the optimum temperature, time of incubation and humidity conditions had to be standardized. It appeared that the greatest variable affecting the silylation process was the existing humidity or moisture content in the reaction vessels. It is known that water molecules do compete with the drug molecules for BSTFA. For this reason each tube was thoroughly dried and flushed with dry nitrogen before excess BSTFA was added to the sealed reaction tubes. The optimal reaction time and temperature were 15 min and 60°.

Typical chromatograms of the silylated bases are presented in Figs. 2 and 3. Standards of naltrexone and etorphine were derivatized with BSTFA and chromatographed (Fig. 2A). The peaks at 1.2 and 4.0 min are minor products of the silylation reaction of naltrexone and BSTFA and the peak at 4.3 min is a minor co-derivative of etorphine with BSTFA. The relative retention times of naltrexone and etorphine were 5.1 and 6.6 min, respectively. The chromatogram in Fig. 2B represents naltrexone and etorphine standards added to 2.0 ml of blank urine, extracted, silylated with BSTFA and chromatographed. It is apparent that the chromatogram is reasonably free of interfering peaks. Using control urines from five individuals indicated the absence of major interfering peaks at any zone on the chromatogram under identical GLC conditions. Fig. 3A represents a chromatogram which was prepared from the urine extract of a subject taking 180 mg naltrexone daily. It is apparent that a much larger quantity of beta-naltrexol than naltrexone is present in the urine of patients

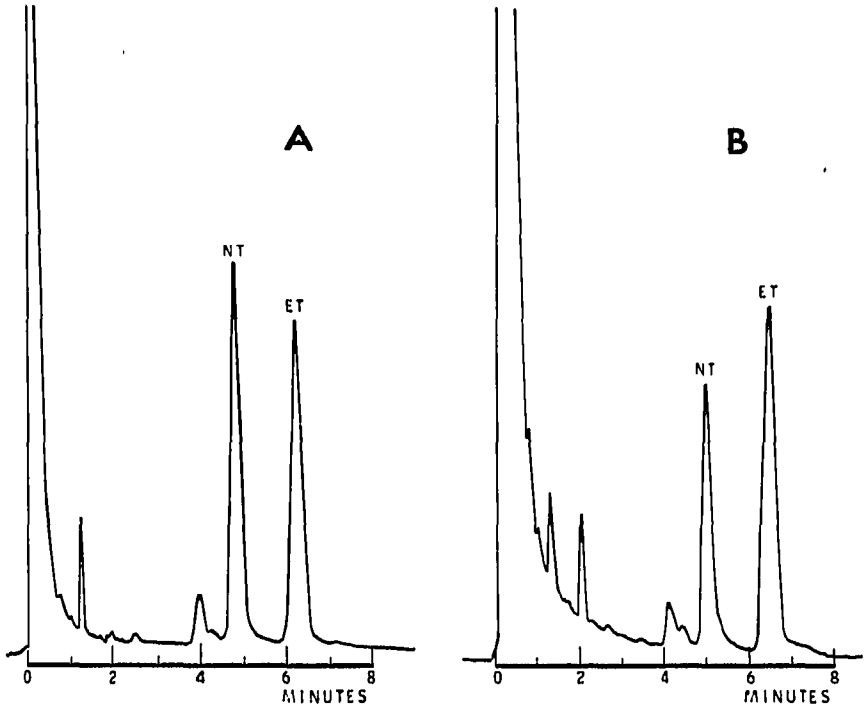


Fig. 2. Chromatograms of standards of naltrexone (NT) and etorphine (ET). (A), Standards derivatized with BSTFA. (B), Standards added to urine, extracted and derivatized with BSTFA.

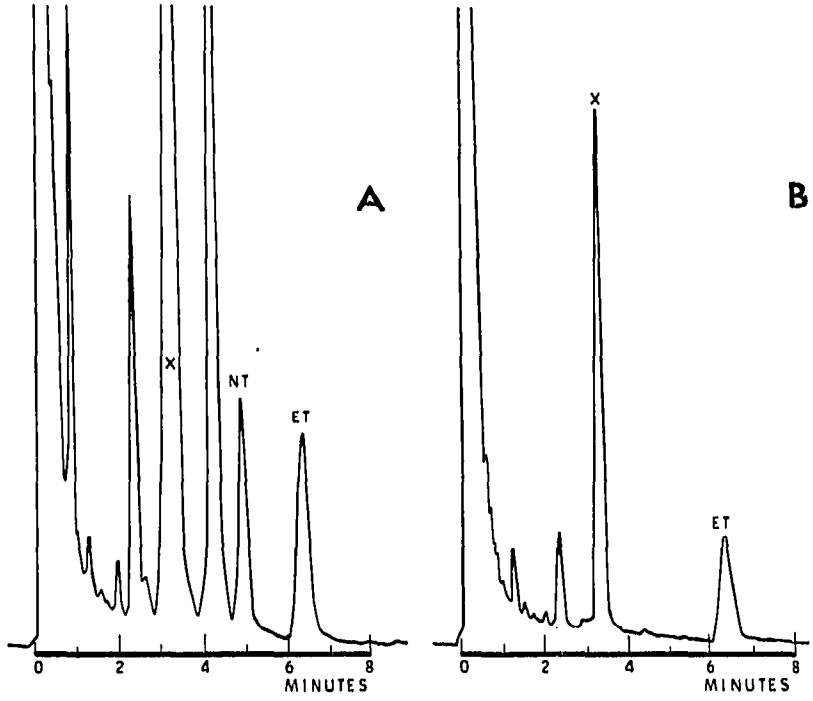


Fig. 3. (A), Sample chromatogram of the BSTFA derivative from the urine extract of a patient treated with 160 mg naltrexone (NT) daily. (B), BSTFA derivative of beta-naltrexol (X) and etorphine (ET) standards.

treated with naltrexone. The large peak at 4.3 min covering the minor co-derivatives of naltrexone and etorphine is not as yet identified.

Beta-naltrexol standards were initially provided by Dr. Cone but later larger quantities were isolated in our laboratory from the urine of patients who were taking large doses of naltrexone daily (see Materials and methods for isolation procedure). The preparative thin-layer chromatographic system provided a clean separation between naltrexone (R_F 0.82) and beta-naltrexol (R_F 0.20). A GLC scan of beta-naltrexol isolated from human urine and added etorphine following silylation with BSTFA is shown in Fig. 3B. The two peaks at 1.3 and 2.4 min are minor co-derivatives of beta-naltrexol and BSTFA. The complete absence of naltrexone (at 5.1 min) indicates the purity of the isolated metabolite from the parent compound.

In order to provide a quantitative method in which derivatization is a necessary step, one should be certain that minor peaks are formed at a constant rate, independent of the concentration of the bases present. We examined the minor peak of naltrexone at 4.0 min, using the concentrations of naltexrone ranging between 2 and 8 μg in the reaction mixture. The results in Table I indicate that the peak at 4.0 min was approximately 14% of the major peak (5.1 min) and was independent of the concentration of naltrexone present in the reaction mixture. This information and the finding that the standard calibration curves are routinely reproducible indicate that under standardized conditions the conversion ratios between major and minor peaks of silylated naltrexone, beta-naltrexol and etorphine are constant.

TABLE I

DERIVATIZATION OF NALTREXONE FREE BASE (NT) WITH BSTFA

Incubation at 60° for 15 min. RRT = relative retention time.

Amount NT derivatized (μg)	*Minor peak (mm) (RRT = 4 min)*	*Major peak (mm) (RRT = 5.1 min)*	*Ratio minor peak:major peak*
2	8	56	0.143
4	13	97	0.134
6	21	144	0.146
8	29	210	0.138

The ranges of the standard curves prepared (see Materials and methods) are practical concentrations for the determination of the bases in unknown human urine samples and do not represent the lowest range of sensitivity attainable for the silylated derivatives of naltrexone and beta-naltrexol. The absolute sensitivity of this method is between 0.02 and 0.01 μg/ml of urine for both naltrexone and beta-naltrexol.

Quantitative analysis of naltrexone and its metabolites was performed to determine the urinary excretion profile of naltrexone in man. Twenty-four-hour urine samples were collected from three volunteers who were chronically taking 180 mg naltrexone per day. The volunteers were out-patients thus the absolute accuracy of the 24-h collection is not known. To ascertain that the urines submitted were indeed 24-h collections, creatinine levels were determined. The expected 24-h creatinine output in a healthy adult male is 20–26 mg per kg body weight. Subjects 1, 2 and 3 excreted 26.3 mg/kg, 22.7 mg/kg and 26.7 mg/kg, respectively.

Before extraction the samples were split. One set was extracted unhydrolyzed and the other set was hydrolyzed in order to liberate the conjugated metabolites. Both Glusulase and acid hydrolysis yielded identical results. Difference in the quantity of naltrexone and beta-naltrexol in the same patients urine between the unhydrolyzed and hydrolyzed samples yield the fraction of the metabolite which is conjugated.

The urinary excretion data on naltrexone are presented in Table II. The average free naltrexone excreted in the urine was 0.81 mg per 24 h and the conjugated fraction was 8.26 mg per 24 h indicating that approximately 90% of the parent compound is excreted in the conjugated form. In this group of subjects the average calculated recovery of naltrexone was only 5% of the administered dose ranging between 9.5% and 2.0%. In Table III the excretion pattern of beta-naltrexol is presented. The average free beta-naltrexol excreted in urine was 62.26 mg per 24 h and the conjugated fraction was 25.20 mg per 24 h. Approximately 70% of the metabolite was excreted free and 30% in the conjugated form. In the three subjects studied the free and conjugated beta-naltrexol accounted for an average of 50% of the administered dose in a 24-h period.

TABLE II

RECOVERY OF NALTREXONE (NT) FROM THE 24-h URINE OF SUBJECTS STABILIZED ON 180 mg NALTREXONE DAILY

Subject	*Free NT (mg)*	*Conjugated NT (mg)*	*Total NT (mg)*	*Percent of dose*
1	1.81	15.35	17.16	9.5
2	0.27	3.19	3.46	1.9
3	0.36	6.25	6.61	3.7
Mean	0.81	8.26	9.08	5.0
Percent of total	8	92		
Percent of administered dose	0.4	4.6		

The urinary excretion profile of naltrexone reported here is in close agreement with the findings of Cone *et al.*[6]. Their study, as well as ours, indicates that in man beta-naltrexol is the major biotransformation product which in fact may be responsible for the long duration of narcotic antagonistic action of naltrexone. This is suspected also

TABLE III

RECOVERY OF BETA-NALTREXOL (X) FROM THE 24-h URINE OF SUBJECTS STABILIZED ON 180 mg NALTREXONE DAILY

Subject	*Free X (mg)*	*Conjugated X (mg)*	*Total X (mg)*	*Percent of dose*
1	53.22	18.91	72.13	40.0
2	65.98	26.22	92.20	51.2
3	67.60	30.47	98.07	54.5
Mean	62.26	25.20	87.46	48.6
Percent of total	71	29		
Percent of administered dose	34.6	14.0		

from our preliminary findings which show that beta-naltrexol is the primary circulating metabolite present in plasma. Since the urinary excretion of naltrexone is greatly dependent on biotransformation before its elimination into the urine, individual differences in drug biotransformation rates may affect the pharmacological action of naltrexone in various individuals. The relative potency of naltrexone and beta-naltrexol in man has not been determined yet, thus one cannot predict the significance of rapid or slow metabolism of naltrexone in man.

In conclusion it can be stated that the small percentage recovery of the naltrexone dose as the parent compound and the great abundance of beta-naltrexol in the 24-h urine indicate that biotransformation is an important pre-requisite for the elimination of naltrexone from the body.

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