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Note

Determination of 6-β-naltrexol and naltrexone by bonded-phase adsorption thin-layer chromatography

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Naltrexone is a long-acting opiate receptor blocking agent, marketed under the trade name of Trexan®. It is used for the prevention of readdiction of ex-opiate addicts to heroin after detoxification [1—4]. Naltrexone has no readily observable physiological or pharmacological effects. Its presence in the body is acknowledged only when an opiate agonist (e.g. heroin) is administered and its typical effects of euphoria and analgesia are blocked [5]. Success of naltrexone therapy depends on the regular intake of naltrexone by the subjects once every two to three days. There is little if any motivation for the subjects to take naltrexone other than their desire to remain opiate-free or the same desire of their friends and close family members. Naltrexone serves as an "insurance policy". When it is taken regularly, the subjects cannot enjoy heroin nor can they become dependent on opiates. Monitoring whether or not the naltrexone therapy is in effect is best achieved by urine analysis of the subjects for the major metabolite of naltrexone, 6-β-naltrexol.

Currently, the primary methodology for naltrexone and 6-β-naltrexol detection is gas chromatography (GC) [6—9]. These GC methods are expensive and

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time-consuming and therefore not practical for routine clinical urine testing. Treatment professionals need a simple and inexpensive test to monitor naltrexone compliance. In this paper we describe a method which can easily be adopted by analytical laboratories to monitor urine samples for the presence or absence of 6-β-naltrexol and naltrexone.

EXPERIMENTAL

Materials

Naltrexone and 6-β-naltrexol were gifts from Du Pont de Nemours (Wilmington, DE, U.S.A.). Toxi-Prep™ extraction columns (Amberlite XAD-2 minibed columns), column activator, elution solvent, Multi-Prep extraction system manifold and Multi-Prep thin-layer chromatography (TLC) spotter were purchased from Biochemical Diagnostics (Farmingdale, NY, U.S.A.).

Drug standards, to test possible interference, were also purchased from Biochemical Diagnostics. Methanol, ethyl acetate and acetone were reagent or HPLC grade, and were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.), as was the chloroplatinic acid. Sodium nitrite, reagent grade, was purchased from Crescent (Hauppauge, NY, U.S.A.). Glusulase was purchased from Endo Labs. (Wilmington, DE, U.S.A.).

Iodoplatinate solution was prepared by adding 1 g of chloroplatinic acid to 50 ml of water. In a separate container 10 g of potassium iodide were added to 100 ml of water. The two solutions were mixed and brought to a final volume of 1000 ml.

Equipment and chemicals for Toxi-Lab® system were purchased from Fred Morrow Assoc. (Palisades, NJ, U.S.A.). TLC plates (S:250F) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Samples

Naltrexone-positive urine samples (30 ml) were collected from patients who were medicated with naltrexone. Negative urine samples were obtained from staff members. The urine samples were taken from patients during their clinic visits, usually 24, 48 or 72 h after the last dose. These collection times were chosen because they were representative of typical clinic schedules. The urine samples were collected without preservatives and were kept frozen (—11°C) until analyzed. A selected group of samples were kept at room temperature (25°C) for four days. No degradation of 6-β-naltrexol was observed. Thus, while samples are in transport, the lack of refrigeration or freezing does not effect sample integrity.

Extraction

The urine samples were allowed to reach room temperature. The Toxi-Prep columns were activated by pouring through 5 ml of carbonate buffer, pH 9.2, containing 7% "column activator". Urine (20 ml) was poured through the columns. The vacuum pump was turned on and the pressure adjusted to 5—7.6 cmHg. After all the urine was filtered the vacuum pressure was increased to 38—51 cmHg and 10 ml of 15% methanol in water were poured through to remove urinary pigments from the columns. The vacuum was maintained for 10 min to remove all residual water from the columns.

Elution and spotting

The columns were transferred to the spotting rack along with a clean column containing reference standards of naltrexone and 6-β-naltrexol (1 μg each). All columns were attached to sample applicator tips of the Toxi-Prep system. The elution solvent was ethyl acetate—isopropanol (85:15); 1.5 ml of this solution were added to the columns and allowed to flow through by gravity onto the TLC plates. A heating unit of the Toxi-Prep system upon which the TLC plates were placed kept the temperature at 80°C during spotting and insured rapid evaporation, resulting in small circular spots of less than 1 cm in diameter. After spotting, using an additional 1.0 ml of eluting solvent, the remaining 6-β-naltrexol was washed off the column into siliconized test tubes. These samples were used for GC confirmation for the presence of 6-β-naltrexol.

TLC development and visualization

Developing was performed in standard size glass tanks of 10 × 23 × 23 cm. The developing solvent was ethyl acetate—methanol—ammonium hydroxide—water (170:20:4:6). The solvent (200 ml) was added to each tank. Saturation pads were placed in the tanks, the tanks were covered and allowed to equilibrate for 10—15 min. Development of the plates was allowed until the solvent front reached a prescored line 14 cm above the origin. After removal the plates were air-dried, followed by drying in an oven at 100°C for 5 min. The plates were sprayed with a solution of 2% sodium nitrite in 1.5 *M* hydrochloric acid. After drying at room temperature, yellow spots were visualized at R_F 0.34 for 6-β-naltrexol and R_F 0.54 for naltrexone (if present). The detection could be intensified by observation of the spots of 6-β-naltrexol under UV light (254 nm). Both naltrexone and 6-β-naltrexol were fluorescent yellow.

Further assurance of identification of 6-β-naltrexol and naltrexone can be achieved by using a second color developing spray, iodoplatinate. The iodoplatinate reagent changes the color of naltrexone and 6-β-naltrexol spots to purple.

Standards

Standards of 6-β-naltrexol and naltrexone were placed on TLC plates at concentrations of 0.5, 1.0, 2.0, 4.0 and 8.0 μg. The chromatograms were developed and the spots were identified as described above. A concentration-dependent color increase was observed, providing semi-quantitative capability especially if a scanning densitometer is used.

Hydrolysis

We have compared enzymatic, alkaline and acid hydrolysis to liberate 6-β-naltrexol from glucuronic acid conjugation. The comparison was made to determine the most practical and cleanest procedure for hydrolysis, extraction and chromatographic separation of 6-β-naltrexol. Alkaline hydrolysis provided the cleanest and quickest results, while acid hydrolysis was the dirtiest and glusulase hydrolysis the longest of the three procedures. Thus, we adopted alkali hydrolysis.

Basic hydrolysis was performed by adding 0.7 ml of 50% sodium hydroxide to 20 ml of urine. This was heated in a water bath of 100°C for 15 min. After

cooling the pH was adjusted to approx. 9.2, to match the pH of the column activator, by adding 0.5 ml of concentrated hydrochloric acid and 500 mg solid sodium bicarbonate. No loss of 6-β-naltrexol or naltrexone was noted during alkali hydrolysis. The hydrolyzed urine was taken through the extraction procedure as described above.

DISCUSSION

The rationale for the development of this clinical screening method is based on naltrexone metabolism and excretion patterns in human subjects [10—12]. It is known that naltrexone constitutes only 20% of the total base recovered in a 24-h urine collection and 90% of naltrexone is conjugated [11]. Thus, screening for the parent compound (naltrexone) is not suited for determination of compliance, because too little of naltrexone is excreted, and even that occurs too early after the dose. 6-β-Naltrexol is the major urinary excretion product of naltrexone and is present in significant quantities [10—12]. Approximately 70% of the total base was recovered from urine as free 6-β-naltrexol and only 30% was conjugated to glucuronides [11]. These data suggested that for a practical qualitative clinical test the monitoring of 6-β-naltrexol was best suited. It is present in sufficient concentrations in the free form. The lower limit of sensitivity of the TLC system for 6-β-naltrexol and naltrexone was only about 0.5 μg. The figures in Table I were calculated from a previous study and represent the average data from six subjects [10]. It serves as a guide to estimate the amount of 6-β-naltrexol in the urine at various periods after the dose. There is no apparent problem of detection up to 72 h. The estimation indicates 10 μg of 6-β-naltrexol present in 20 ml of urine at 48—72 h. At 72 h the excretion of 6-β-naltrexol closely approaches the sensitivity of the test. Rapid eliminators of naltrexone may have negative urines by the TLC at 72 h. In some cases, glusulase hydrolysis of the 72-h samples may

TABLE I

URINARY EXCRETION OF FREE 6-β-NALTREXOL (β-OL) AFTER 50 mg ORAL DOSES

Data from ref. 10.

Time (h)	Amount of free β-OL per time period (mg)	Volume of estimated urine output* (ml)	Concentration of free β-OL (μg/ml)	Amount of total free β-OL from 20 ml urine (μg)
0—2	2.60	180	14.4	288
2—4	1.60	200	8.0	160
4—8	1.80	250	7.2	144
8—16	2.75	375	7.3	146
16—24	1.10	375	2.9	58
24—36	1.65	750	2.7	44
36—48	0.65	750	0.9	18
48—72	0.75	1500	0.5	10

*Estimated fractional urine volumes from ref. 11.

TABLE II

DETECTION OF FREE 6-β-NALTREXOL IN URINE BY TLC AND CONFIRMATION BY TOXI-LAB AND GC

Sample*	Time of collection	Results		
		Bonded-phase adsorption TLC	Toxi-Lab	GC (μg/ml)
1	24	+	+	9.5
2	24	+	+	10.2
3	48	+	+	4.8
4	24	+	+	8.0
5	48	+	+	3.8
6	Negative	—	—	0.0
7	48	+	+	3.5
8	24	+	+	8.7
9	Negative	—	—	0.0
10	72	+	—	0.8
11	48	+	+	4.8
12	72	+	—	0.4

*Naltrexone-positive urine samples. The sample order was mixed arbitrarily and analyzed in a double-blind fashion.

be necessary to bring the levels of 6-β-naltrexol close to the detection sensitivity of the method. If the sample is still negative by TLC after hydrolysis, GC analysis may be performed to confirm the absence of 6-β-naltrexol. The sensitivity of the GC method for both naltrexone and 6-β-naltrexol is 20 ng/ml.

Commonly abused drugs such as quinine (heroin diluent), oxazepam, diazepam, cocaine, amphetamine, codeine, morphine, meperidine, phenothiazines, barbiturates and glutethemide were tested in the system and did not interfere with 6-β-naltrexol and naltrexone analysis. Nicotine, present in many urine samples of smokers, migrates just above naltrexone on the TLC plate. It may serve as a marker for naltrexone if they are both present. Caffeine- and theophylline-containing products are widely used but they did not interfere with the procedure.

Toxi-Lab and a more sensitive reference GC method [6] were compared with the bonded-phase adsorption TLC method described in this paper. The data in Table II indicate excellent agreement among the three methods. The Toxi-Lab procedure was somewhat less sensitive than the bonded-phase adsorption TLC procedure as indicated by the negative results of the 72-h samples.

In conclusion, a urinalysis test is described for 6-β-naltrexol, utilizing commercially available columns, plates, solvents and equipment. Tests can be performed quickly and economically. Thus, laboratory analysis of naltrexone compliance can be easily monitored. Up to 48 h after the naltrexone dose the test is highly reliable using unhydrolyzed urine. Hydrolysis may be necessary of urine samples taken more than 40 h after the dose to bring the concentration of free 6-β-naltrexol to the bonded-phase adsorption TLC methods detection limit.

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