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^{63}Ni ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY FOR BUPRENORPHINE AND METABOLITES IN HUMAN URINE AND FECES

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SUMMARY

A ^{63}Ni electron-capture gas chromatographic assay is described for buprenorphine, a potent narcotic agonist—antagonist. In addition, the assay is useful for the measurement of the metabolite norbuprenorphine and demethoxybuprenorphine, a rearrangement product resulting when buprenorphine is exposed to acid and heat. An extraction procedure was developed which optimized recovery of buprenorphine from biological samples and produced minimal background interferences and emulsion problems. Extract residues were derivatized with pentafluoropropionic anhydride and assayed by gas chromatography. Samples were analyzed with and without enzyme hydrolysis, thus providing a selective and sensitive assay for both free and conjugated buprenorphine, norbuprenorphine and demethoxybuprenorphine. The lower limits of detection following extraction of a 1-ml sample were ca. 10 ng/ml for buprenorphine and demethoxybuprenorphine and 5 ng/ml for norbuprenorphine. Application of the assay to human samples following a 40-mg oral dose of buprenorphine produced no evidence for the presence of demethoxybuprenorphine in urine or feces. Norbuprenorphine (free and conjugated) was present in urinary and fecal samples; buprenorphine (free and conjugated) was found in high amounts only in feces and in trace amounts in urine as conjugated buprenorphine. The urinary and fecal excretion pattern observed for a human subject following oral dosing of buprenorphine suggests enterohepatic circulation of buprenophrine.

INTRODUCTION

Buprenorphine is a synthetic *endo*-ethanotetrahydrooripavine derivative which has both analgesic and opioid antagonistic properties [1]. As an analgesic it is some 25 to 40 times more potent than morphine, whereas when used as an antagonist it is equivalent in potency to the narcotic antagonist naltrexone. The relatively low toxicity of buprenorphine, minimal abstinence

syndrome on abrupt withdrawal following chronic administration and suppression effects on heroin self-administration [2] led to the proposal that it be used for the chemical maintenance of heroin addicts [2, 3].

Because of the high potency of buprenorphine, assay methods for measurements of drug in plasma and cerebrospinal fluid have been based on sensitive radioimmunoassay [4] and radioreceptor [5] techniques. These methods lack specificity, however, and an assay was needed which would simultaneously measure parent drug, potentially active metabolites and drug artifacts in biofluids. This report describes the development of a ^{63}Ni electron-capture gas chromatographic (GC) assay for buprenorphine, norbuprenorphine and the acid-catalyzed rearrangement product, demethoxybuprenorphine (Fig. 1) in human urine and feces. Biological samples collected from subjects participating in assessment studies of buprenorphine as a treatment drug for narcotic addiction were assayed by these methods.

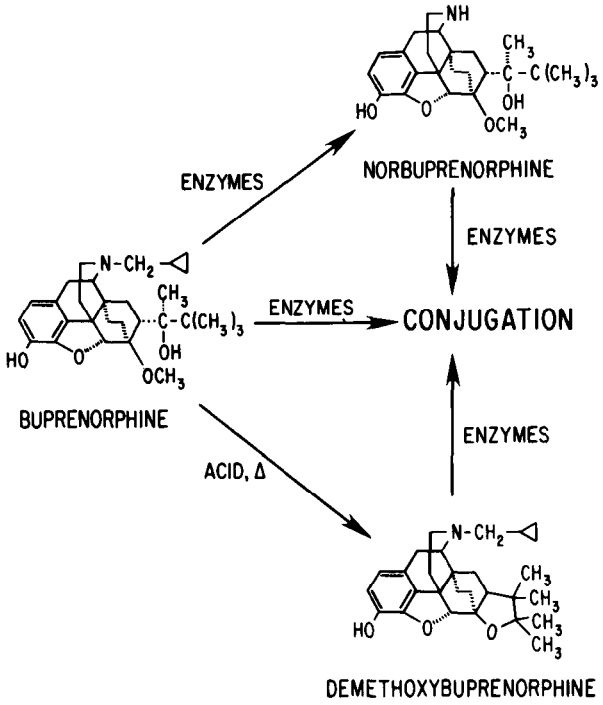

Fig. 1. Buprenorphine biotransformation products and acid-catalyzed rearrangement to demethoxybuprenorphine.

MATERIALS AND METHODS

Standards and reagents

Drug standards were obtained from the following sources: buprenorphine, [15,16-$^3H_2$]buprenorphine (specific activity 38 Ci/mmol, > 95% purity), etorphine (internal standard, Research Technology Branch, Division of Research, National Institute on Drug Abuse, Rockville, MD, U.S.A.); norbuprenorphine (Reckitt and Coleman, Hull, U.K.). The acid-catalyzed

rearrangement product of buprenorphine, demethoxybuprenorphine (N-cyclopropylmethyl-6,14-*endo*-ethano-2′,3′,4′,5′,7,8-hexahydro-4′,4′,5′,5′-tetramethylfurano[2′,3′:6,7]normorphide, DMB) was synthesized from buprenorphine as reported [6]. The identity and purity of these compounds were established by gas chromatography—mass spectrometry and thin-layer chromatography prior to their use. Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockville, IL, U.S.A.). All other chemicals were of reagent grade quality.

Gas chromatography

A Perkin-Elmer gas chromatograph Model Sigma 2 equipped with a ^{63}Ni electron-capture detector was used for the analyses. A glass column (1.8 m × 2 mm I.D.) was packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh). The column was operated isothermally at 260°C. Other conditions were as follows: detector temperature, 320°C; injector temperature, 275°C, carrier gas flow-rate (nitrogen) 20 ml/min : make up gas flow-rate, 40 ml/min.

Subjects, dosing, sample collection

The subjects were healthy, drug-free male volunteers with a history of opiate abuse. Their ages ranged from 21 to 45 years. During the study the subjects were housed in the research ward of the Addiction Research Center (Baltimore, MD, U.S.A.). All gave informed consent to participate in the study, the protocol for which had been approved by the Institutional Review Board of Baltimore City Hospitals. Buprenorphine or placebo was administered orally (10—40 mg) or subcutaneously (1—2 mg) every four days. Urine and feces were collected throughout the study and frozen until time of assay.

Sample preparation and hydrolysis

Frozen fecal samples were weighed and placed in 1.0 l of methanol. The mixture was stirred overnight and decanted into centrifuge tubes. Following centrifugation, aliquots (1.0 ml) were removed and evaporated to dryness under a stream of nitrogen at 40°C.

For enzyme hydrolysis, 1.0 ml of buffer (2 *M* sodium acetate, pH 5.2) was added to the fecal residue or urine sample (1.0 ml) followed by the addition of etorphine, internal standard (I.S., 200—250 ng) and enzyme solution (Glusulase®, Endo Labs., Garden City, NY, U.S.A.) containing 30,000 U of glucuronidase and 6000 U of sulfatase. The samples were mixed and incubated at 37°C for 20 h. Following the incubation period, the reaction was stopped by the addition of 1 ml of 10 *M* phosphate buffer, pH 10.

Extraction and derivatization of buprenorphine and metabolites

Enzyme hydrolyzed or untreated urinary and fecal samples (1.0 ml) were placed in 15-ml centrifuge tubes. An aqueous aliquot of I.S. (100 μl, 200—250 ng) was added to those samples which had not been hydrolyzed. For standard curves, 1.0-ml aqueous aliquots of mixtures of buprenorphine (10—1000 ng/ml), norbuprenorphine (25—2000 ng/ml) and demethoxybuprenorphine (25—2000 ng/ml) were added. The extraction procedure, as outlined in Fig. 2, began with the addition of buffer (1.0 ml of 10 *M* potassium phosphate, pH

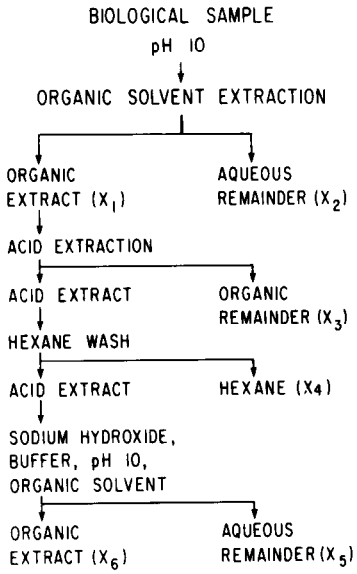

Fig. 2. Extraction procedure for buprenorphine and metabolites from biological samples.

10), and the final pH was adjusted to 10.0 ± 0.1 with 1 *M* sodium hydroxide. Ethyl acetate—heptane (6 ml, 4:1, v/v) was added and the contents were vortexed for 30 sec. For enzyme-hydrolyzed samples, the tubes alternatively were gently shaken on a bench shaker for 30 min in order to prevent emulsion formation. The samples then were centrifuged and the organic layer was transferred to a clean tube. The extraction was repeated and the combined organic extracts were treated with 3 ml of cold 0.1 *M* sulfuric acid. This mixture was vortexed for 30 sec and centrifuged. The organic phase was discarded and the acid extract was treated with 6 ml of hexane. After vortexing, the hexane layer was discarded and the pH adjusted to pH 10 ± 0.1 with 1 *M* sodium hydroxide followed by 1 ml phosphate buffer. Heptane—ethyl acetate (6 ml, 1:5, v/v) was added and the contents were vortexed for 30 sec. After centrifugation the organic layer was transferred to a 13-ml centrifuge tube and evaporated to dryness under a stream of nitrogen.

Derivatization of the residue was accomplished by the addition of toluene (100 μl) and PFPA (20 μl) and incubation at room temperature for 1 h. The excess reagent was removed by evaporation under a stream of nitrogen at room temperature. Ethyl acetate (100 μl) was added and an aliquot (1—2 μl) was withdrawn for GC analysis.

Quantitation of buprenorphine and metabolites

Daily standard curves were constructed for buprenorphine, norbuprenorphine and demethoxybuprenorphine. The curves were prepared from the analyses of standard control solutions containing I.S. (250 ng) and buprenorphine, norbuprenorphine and demethoxybuprenorphine added in equal concentrations in ranges of 10—100, 25—250 or 500—2000 ng/ml. The standard solutions were processed and analyzed in the same manner as drug specimens. Linear responses for peak height ratios versus concentration were

obtained for all concentration ranges with $r \geqslant 0.98$. The lower limits of detection were approx. 10 ng/ml for buprenorphine and demethoxybuprenorphine and 5 ng/ml for norbuprenorphine.

RESULTS AND DISCUSSION

Assay development

During the initial stages of assay development for buprenorphine in biological samples, a variety of experimental conditions were tested and optimized. An extraction scheme (Fig. 2) was developed for the recovery of drug and extractable metabolites from urinary and fecal material. Initial extraction with an organic solvent followed by back-extraction into acid, hexane wash and re-extraction provided a purified drug extract with minimal background interferences when assayed by ^{63}Ni electron-capture GC. The losses and recoveries incurred in the extraction scheme (Fig. 2) with a variety of organic solvents are shown in Table I. The percentages are based on the amount of [^{3}H]buprenorphine added initially and are adjusted for aliquot loss. The percentages X_1 through X_6 reflect the efficiency or loss of each step but also reflect prior losses. Ethyl acetate was slightly superior to methylene chloride in the initial extraction step, X_1, for the recovery of buprenorphine from urine at pH 10. The addition of isopropanol improved initial recoveries (X_1) to about 80%. Other solvent combinations, e.g. pentane—isopropanol (9:1) and ethyl acetate—heptane (4:1) produced moderate (49%) to good (69%) initial recoveries, respectively. Acid extraction of buprenorphine from pentane—isopropanol (9:1) was most efficient (X_3 = 1.6, indicating a small loss), whereas moderate losses occurred with the ethyl acetate based solvents (X_3 ranged from 12.5% to 21.8%) and substantial losses occurred with methylene chloride based solvents (X_3 ranged from 30.1% to 53.4%). Following acid-extraction the hexane wash step (X_4) produced little loss (ca. 1%) in all cases. Recoveries in the final extraction step (X_6) reflect losses in all prior steps as well as losses to the aqueous phase (X_5) and indicate the overall efficiency of the entire

TABLE I

PERCENTAGE RECOVERY AND LOSSES OF [^{3}H]BUPRENORPHINE DURING EXTRACTION

The recoveries reflect the efficiency of each step and that of all prior steps of the extraction scheme (Fig. 2). The percentages ± standard error are based on the amount of [^{3}H]buprenorphine initially added. Corrections were not made for that portion of solvent miscible in the other phase, hence the sum of X_1 and X_2 does not equal 100%.

Solvent (ratio, v/v)	Fraction					
	X_1	X_2	X_3	X_4	X_5	X_6
Ethyl acetate	73.6 ± 0.3	20.7 ± 0.2	16.8 ± 0.1	1.2 ± 0.1	6.7 ± 0.6	47.5 ± 0.6
Ethyl acetate—isopropanol (1:9)	80.2 ± 0.4	15.2 ± 0.4	21.8 ± 0.4	1.2 ± 0.1	3.7 ± 0.1	52.8 ± 0.6
Ethyl acetate—heptane (4:1)	69.2 ± 0.8	23.6 ± 0.5	12.5 ± 0.2	1.2 ± 0.1	6.7 ± 0.6	44.5 ± 1.0
Methylene chloride	56.0 ± 0.5	29.2 ± 0.8	30.1 ± 2.2	1.3 ± 0.2	3.4 ± 0.3	18.9 ± 2.1
Methylene chloride—isopropanol (7:3)	82.3 ± 1.5	12.9 ± 0.2	53.4 ± 1.6	1.3 ± 0.1	2.6 ± 0.1	34.0 ± 0.4
Pentane—isopropanol (9:1)	49.0 ± 3.6	40.7 ± 2.1	1.6 ± 0.1	1.3 ± 0.1	5.6 ± 0.6	28.1 ± 2.4

process. The highest recoveries were obtained with the ethyl acetate based solvents with efficiencies ranging from 44.5% to 52.8%. Ethyl acetate—heptane (4:1) was selected as the solvent of choice based on its overall extraction efficiency of 44.5 ± 1.0%, low background contribution from biological control samples and minimum emulsion problems encountered during extraction.

In addition to solvent selection, a number of other factors were optimized for recovery of buprenorphine from biological samples. During extraction it was found that vortexing samples for 30 sec rather than mechanical shaking for 15 min resulted in slightly higher recoveries. However, severe emulsion problems occurred upon vortexing enzyme-hydrolyzed samples and mechanical shaking for 30 min was substituted in the processing of these samples.

The effect of pH on extraction efficiency was determined across the pH range 7—12. Only small differences in recoveries were found across the entire range and pH 10 was selected as the pH of choice.

Conditions suitable for the complete derivatization of buprenorphine to the mono-PFPA derivative were found to be incubation at room temperature (22—26°C) with 25 μl PFPA in 100 μl of toluene. Heating during derivatization was found to produce extensive decomposition of both buprenorphine and norbuprenorphine and was avoided.

Prior to the completion of the assay development procedure for buprenorphine and metabolites, it was necessary to establish that buprenorphine and metabolites were completely stable under the selected conditions. Buprenorphine has been shown previously to undergo rearrangement in the presence of acid and heat to demethoxybuprenorphine [6]. At pH ≤ 1 and in the presence of heat, the rearrangement reaction can approach total conversion to demethoxybuprenorphine. However, at pH 1 in the absence of heat, i.e. room temperature, aqueous buprenorphine solutions were shown to be stable up to ten weeks. Thus, in the current extraction procedure, brief exposure of buprenorphine to cold 0.1 *M* sulfuric acid was shown not to produce detectable amounts of demethoxybuprenorphine. Therefore it was possible with this procedure to determine if demethoxybuprenorphine is produced by humans in vivo rather than as an artifact of the assay procedure.

Search for demethoxybuprenorphine in drug samples

Following extraction and derivatization, buprenorphine, norbuprenorphine and demethoxybuprenorphine could be separated, detected and measured with reference to an internal standard by GC (Fig. 3). The stronger response obtained for norbuprenorphine (Fig. 3A) was due to formation of a di-PFPA derivative as opposed to formation of mono-PFPA derivatives for the other compounds. The assay procedure produced no interferences from control samples (Fig. 3D) or other tetrahydrooripavine derivatives and had sufficient sensitivity to measure demethoxybuprenorphine down to ca. 10 ng/ml (Fig. 3C).

Analyses of urine samples following large oral doses of buprenorphine produced no evidence of demethoxybuprenorphine formation and excretion. Fig. 3B is a typical chromatogram of an extract of unhydrolyzed urine obtained from a human subject 8—12 h following a 40-mg oral dose of buprenorphine. The arrows indicate the retention times at which demethoxy-

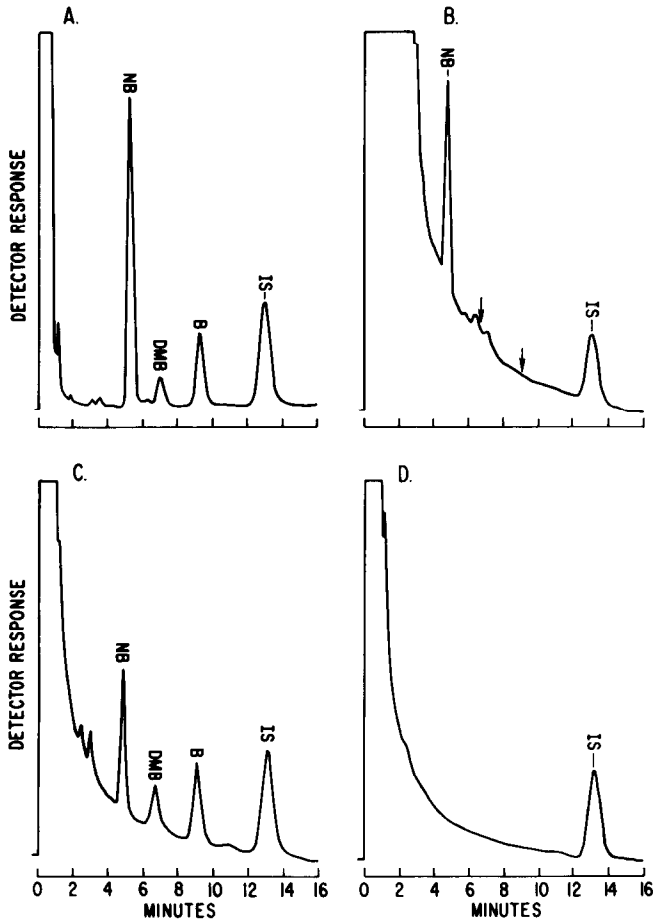

Fig. 3. Gas chromatograms of standards of buprenorphine and metabolites and extracts from human urine. (A) Unextracted standards (1 ng on column); (B) extract of unhydrolyzed urine collected 8—12 h following a 40-mg oral dose of buprenorphine (arrows indicate retention times of demethoxybuprenorphine and buprenorphine); (C) extract of control urine with standards added (NB, DMB and B 100 ng. IS, 200 ng); (D) extract of control urine. All samples were derivatized with PFPA. Peaks: NB = norbuprenorphine; DMB = demethoxybuprenorphine; B = buprenorphine; and IS = etorphine, internal standard.

buprenorphine and buprenorphine would have emerged. Demethoxybuprenorphine was also not present in samples collected at other time periods nor was it detectable following buprenorphine administered by other routes. It should be noted, however, that although demethoxybuprenorphine was not excreted by these subjects, the conversion of buprenorphine to this biologically active compound [7] either by the acidic environs of the stomach or by enzymatic processes cannot be ruled out completely since only a small portion of the administered dose of buprenorphine could be recovered. The possibility exists that demethoxybuprenorphine was biotransformed into other products which were not detectable by this assay.

Excretion of buprenorphine and norbuprenorphine

Human urinary and fecal samples were assayed for buprenorphine and norbuprenorphine by the described assay procedure. Samples were analyzed untreated (free) and following enzyme hydrolysis (total) to provide measures of conjugated metabolites. Fig. 4 illustrates the typical findings for most samples. Fig. 4A is the response for a control sample with 100 ng and 50 ng added, respectively, of norbuprenorphine and buprenorphine. Responses were linear and reproducible down to ca. 10 ng/ml buprenorphine and 5 ng/ml

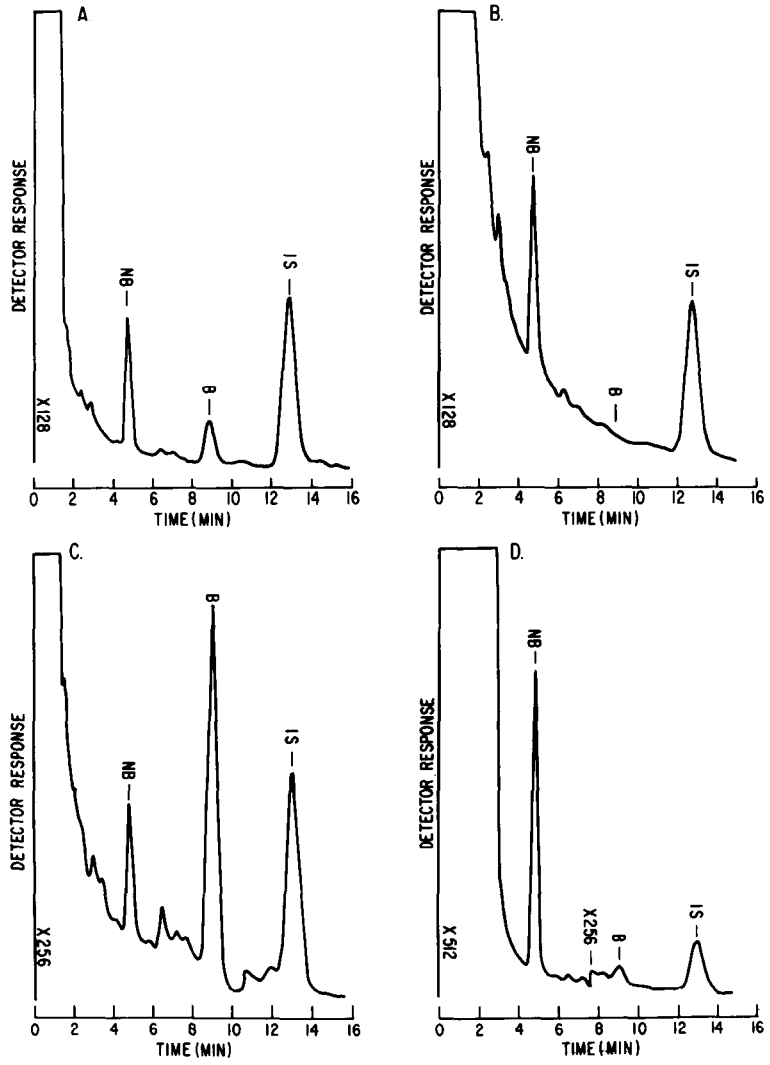

Fig. 4. Gas chromatograms of human biological extracts. (A) Control urine with standards added NB, 100 ng; B, 50 ng; and IS, 250 ng; (B) urine collected 48—60 h following a 40-mg oral dose of buprenorphine; (C) hydrolyzed feces sample collected 73 h following a 20-mg oral dose of buprenorphine; (D) urine, same as (B), hydrolyzed with enzyme. All samples were derivatized with PFPA. Peak: Nb = norbuprenorphine; B = buprenorphine; and IS = etorphine, internal standard.

norbuprenorphine. All of the urinary analyses were consistent in that free buprenorphine was not detectable at any time period (e.g., Fig. 4B) and conjugated buprenorphine was present in very small amounts (e.g., Fig. 4D). Both free and conjugated norbuprenorphine were present at all time periods tested through six days following drug administration. In contrast to the results from urinary analyses, fecal analyses showed larger amounts of free parent buprenorphine present than the metabolite norbuprenorphine (Fig. 4C). It should be noted that this was occurring at time periods during which norbuprenorphine was being excreted in urine in much larger amounts than total buprenorphine. These findings indicate the likelihood of enterohepatic circulation of buprenorphine in human similar to that found for buprenorphine in rat [8].

The overall cumulative urinary excretion of conjugated buprenorphine, conjugated norbuprenorphine and free norbuprenorphine for a human subject following a 40-mg oral dose is shown in Fig. 5. The major metabolite, conjugated norbuprenorphine, accounted for only ca. 4% of the administered dose with free norbuprenorphine and conjugated buprenorphine each accounting for ca. 1%. The remainder of the dose could be partially accounted for by elimination in feces. Other metabolites were not detected but could possibly represent a sizable portion of the administered dose.

Overall, this assay clearly established that buprenorphine is excreted intact in feces over a long time course (at least seven days) and as conjugated norbuprenorphine in urine over an equal time period. Detailed investigations of buprenorphine following other routes of administration are underway.

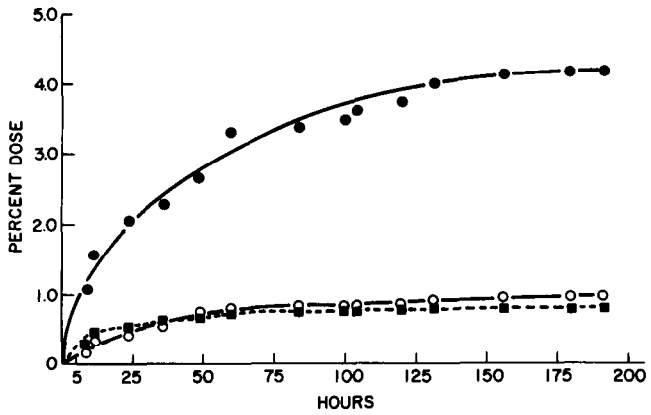

Fig. 5. Cumulative urinary excretion of buprenorphine metabolites following a 40-mg oral dose of buprenorphine to a human subject. ▪, Conjugated buprenorphine; ○, free norbuprenorphine; ●, conjugated norbuprenorphine.

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