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GENERAL PROCEDURE FOR THE ISOLATION AND IDENTIFICATION OF 6-α- AND 6-β-HYDROXY METABOLITES OF NARCOTIC AGONISTS AND ANTAGONISTS WITH A HYDROMORPHONE STRUCTURE

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SUMMARY

In order to aid in the elucidation of the metabolism of drugs containing the hydromorphone structure, a method is described for isolation from urine, separation and identification of the 6-α- and 6-β-hydroxy metabolites. The samples were acid-hydrolyzed, extracted, and separated by thin-layer chromatography. The zone containing the hydroxy metabolites was removed and the compounds were re-extracted and analyzed by gas–liquid chromatography (GLC). Silylation of the extract was necessary in most cases for optimum GLC resolution of the α- and β-hydroxy epimers.

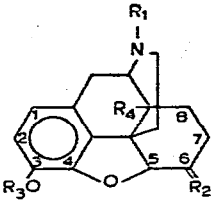
To demonstrate application of this method, the urine of guinea-pigs and rats which had received a single 40-mg dose of naloxone subcutaneously was analyzed. Analysis indicated a α/β ratio of 0.41 for the guinea-pig. In contrast, the amount of 6-α-naloxol found in the urine of the rat was negligible in comparison with the 6-β-hydroxy metabolite, indicating a species difference in the stereospecificity of the drug-metabolizing enzyme.

INTRODUCTION

The hydromorphone structure of the type shown in Table I appears in a number of clinically useful drugs. Included in this list are the narcotic agonists III–VI used principally for analgesia and their antitussive effect and the narcotic antagonists, I and II, used for their ability to reverse narcotic-induced respiratory depression. Additionally, the narcotic antagonists have been proposed for use in the ambulatory treatment of former narcotic addicts[1]. The widespread clinical use of these compounds has stimulated interest in determining the metabolic transformations accorded these compounds in man and various laboratory animal species. Naltrexone (I) and naloxone (II) are reported to be metabolized in man and numerous animal species by glucuronide conjugation at C-3 and/or reduction of the C-6 keto group to the corresponding 6-α- and/or 6-β-hydroxy metabolite[2–8]. Hydromorphone, V (Dilaudid, Dihydromorphinone) was reported to be metabolized in rabbit by glucuronide

TABLE I

DRUGS AND 6-HYDROXY METABOLITES WITH HYDROMORPHONE STRUCTURES



Parent compound	*6-Hydroxy metabolite**	*Parent compound structure*			
		R_1	R_2	R_3	R_4
I, Naltrexone	Ia, 6-α-Naltrexol Ib, 6-β-Naltrexol	CH_2-cyclopropyl	O	H	OH
II, Naloxone	IIa, 6-α-Naloxol IIb, 6-β-Naloxol	$CH_2CH{=}CH_2$	O	H	OH
III, Oxymorphone	IIIa, 6-α-Oxymorphol IIIb, 6-β-Oxymorphol	CH_3	O	H	OH
IV, Oxycodone	IVa, 6-α-Oxycodol IVb, 6-β-Oxycodol	CH_3	O	CH_3	OH
V, Hydromorphone	Va, 6-α-Hydromorphol Vb, 6-β-Hydromorphol	CH_3	O	H	H
VI, Hydrocodone	VIa, 6-α-Hydrocodol VIb, 6-β-Hydrocodol	CH_3	O	CH_3	H

* The structure of the hydroxy metabolite is identical to that of the parent, except $R_2 = <^{H}_{OH}$ for the α-isomer and $R_2 = <^{OH}_{H}$ for the β-isomer.

conjugation and C-6 reduction[9]. Little is known concerning the metabolism of III, IV, and VI.

There appears to be marked stereospecificity of the drug-metabolizing enzyme responsible for the reduction of the 6-keto group of I and II and this specificity is highly species dependent[2,3]. A major differentiation in stereospecificity appears to exist between avian species (6-α-hydroxy metabolite producer)[10] and man, rabbits, guinea-pigs or rats (6-α- and/or 6-β-hydroxy metabolite producer)[2,4]. Also, the degree of stereospecificity (α/β ratio) within the 6-β-hydroxy metabolite producer group may vary between species and within species. This α/β ratio may be of particular importance in view of the difference in qualitative change in activity reported for the 6-α-hydroxy metabolite of naltrexone from that of the C-6 epimer. Both the 6-α- and 6-β-hydroxy metabolites of I and II have significant antagonistic activity, albeit somewhat lower than the parent keto form. However, the change in configuration of the hydroxyl group from the 6-β-orientation to that of the 6-α-orientation evokes the appearance of significant agonistic activity[11,12].

In order to elucidate the metabolism of the compounds with a hydromorphone structure, methods are needed for the isolation and identification of the α- and β-hydroxy forms which are potential metabolites. The major purpose of this paper is to describe general procedures for the extraction of the compounds of interest from

urine and their separation and identification using thin-layer chromatography (TLC) and gas–liquid chromatography GLC. The application of the methods is demonstrated in the analysis of urine from guinea-pigs and rats which had received naloxone (II).

EXPERIMENTAL

Standards

The following gifts are gratefully acknowledged: I, Ia, II, IIa, and III (from Endo Pharmaceutical, Garden City, N.Y., U.S.A.); Ib and IIb (from Dr. Charles Inturrisi, Cornell University); Va, Vb, and VIb (from Drug Addiction Laboratory, University of Virginia); VIa (from Dr. Everette May, NIH). V and VI were obtained from Knoll Pharmaceutical (Whippany, N.Y., U.S.A.) and Wm. S. Merrell (Cincinnati, Ohio, U.S.A.). IIIb and IVb were prepared by reduction of III and IV, respectively, with formanidinesulfinic acid, as described by Chatterjie *et al.*[11] for the reduction of I and II. IIIa and IVa were prepared by reduction of III and IV, respectively, with sodium borohydride in tetrahydrofuran. Both reduction procedures produced the designated isomer with minor contamination by the corresponding epimer (10% or less). All compounds were analyzed by combined gas chromatography–mass spectrometry (GC–MS) for structural verification.

Gas chromatographic conditions

The analyses were performed on a Varian GC Model 2700 gas chromatograph with flame ionization detectors. Twin glass columns (6 ft. × 2 mm I.D.) were employed. Columns a and b were packed with 3% OV-17 and 3% OV-225, respectively, on 100–120 mesh Gas-Chrom Q. Nitrogen, air and hydrogen flow-rates were 30, 200, and 30 ml/min, respectively. The detector and injector temperatures were maintained at 255°. The column temperature was maintained isothermally as shown in Table III. The reported retention times represent an average of triplicate determinations.

Thin-layer chromatographic material

Gelman ITLC Types SG and SA (Gelman, Ann Arbor, Mich., U.S.A.) were used as the TLC medium.

Sample preparation and analyses

Ten milliliters of sample urine with 10% conc. hydrochloric acid were hydrolyzed by autoclaving at 115° and 15 p.s.i. pressure for 20 min. After cooling the sample was treated with 10 *N* NaOH to *ca.* pH 9. Sodium chloride (1 g), 2 ml K_2HPO_4 buffer (40%), and 15 ml chloroform were added and the contents were shaken for 10 min. Following centrifugation the top aqueous layer was discarded and the organic phase was transferred to a clean tube containing 3 ml of 2 *N* HCl. The contents were shaken, centrifuged, and the organic phase was discarded. The remaining aqueous phase was treated with 10 *N* NaOH to *ca.* pH 9 and 0.5 g NaCl, 2 ml K_2HPO_4 buffer (40%) and 15 ml of chloroform were added. The contents were shaken, centrifuged, and the aqueous phase was discarded. The remaining organic phase was transferred to a clean tube, evaporated to dryness under nitrogen, and reconstituted in 0.5 ml of methanol. The resulting solution was applied to the appropriate TLC medium, dried and eluted with chloroform saturated with aqueous ammonia. The outer edges of the chromatogram were cut and sprayed with potassium iodoplatinate for visualization of the

parent compound and its respective hydroxy metabolites. The region containing the hydroxy metabolites was removed (taking care to eliminate the parent drug), cut into squares of *ca.* 0.5 cm, and extracted thrice with 5 ml of methanol. The methanolic extract was filtered, evaporated to dryness and the residue was taken up in 0.1 ml of methanol. The sample could be analyzed directly by GLC or transferred to an acylation tube for derivatization. The derivatized samples were prepared by evaporating the solution to dryness followed by the addition of 0.1 ml of Tri-Sil "Z" (Pierce, Rockford, Ill., U.S.A.). The sample was sealed and heated in an oil bath at 90–95° for 2–3 h. After cooling 1 μl of sample was injected on-column in the gas–liquid chromatograph.

Naloxone urine collection

Six male Wistar rats and six male guinea-pigs were housed in separate stainless-steel cages equipped with urine collector pans. The animals were healthy and had been drug-free for at least four weeks. A single 40-mg dose of II·HCl was administered subcutaneously (s.c.). Control urine was collected prior to drug. Drug urine was collected for two days. The samples were filtered and frozen until the time of analysis.

RESULTS AND DISCUSSION

Prior separation of the α- and β-hydroxy metabolites from the parent keto compound was considered a necessary requirement of the analytical procedure. This was conveniently accomplished on the ITLC systems shown in Table II. In most cases

TABLE II

R_F VALUES OF DRUGS AND 6-HYDROXY METABOLITES WITH HYDROMORPHONE STRUCTURES

The eluting solvent in both systems consisted of chloroform saturated with aqueous ammonia; R_F values are reported as the mean of triplicate determinations.

Compound	*R_F value*	
	ITLC-SG	*ITLC-SA*
I	0.68	0.07
Ia	0.18	0
Ib	0.12	0
II	0.63	0.06
IIa	0.21	0
IIb	0.13	0
III	0.55	0.03
IIIa	0.16	0
IIIb	0.07	0
IV	1.0	0.34
IVa	0.90	0.13
IVb	0.73	0.06
V	0.26	0
Va	0.08	0
Vb	0.04	0
VI	0.70	0.08
VIa	0.59	0.04
VIb	0.40	0.02

ITLC-SG was the preferred substrate for separation, with the exception of the hydroxy metabolites of IV, which were separated on ITLC-SA. The order of elution profile followed the same general pattern without exception, *e.g.*, β-α-parent. Separations of the α- from the β-hydroxy compounds were only partial in contrast to the excellent separation in most cases of both forms from that of the parent keto compound. The entire separation procedure was fast and efficient and could be repeated for extracts of drug combinations added to control urine without complication.

The relative retention times on OV-17 and OV-225 of the free and silanized derivatives of the α- and β-hydroxy metabolites to the parent compound are listed in Table III, along with the corresponding column temperatures. Optimum conditions were sought for the separation of the α- and β-hydroxy metabolites. In most cases the more polar liquid phase, OV-225, provided the best separation with the exception of Va and Vb, which were only partially resolved. A more complete separation for the silyl derivatives of Va and Vb was obtained on OV-17 at 230°. The low volatility of the free forms of compounds I, II, III, and V and their metabolites necessitated their derivatization for GLC analysis. The best separation of IVa and b and VIa and b occurred as the underivatized form on OV-225. The optimum conditions for each compound are indicated in Table III.

The order of GLC elution of the silyl derivatives of I, II, III, and their metabolites on both OV-17 and OV-225 and of IV and VI and their metabolites on OV-225 followed a β-α-parent elution pattern. All of the compounds which were analyzed underivatized and the remainder of the silyl derivatives shown in Table III fol-

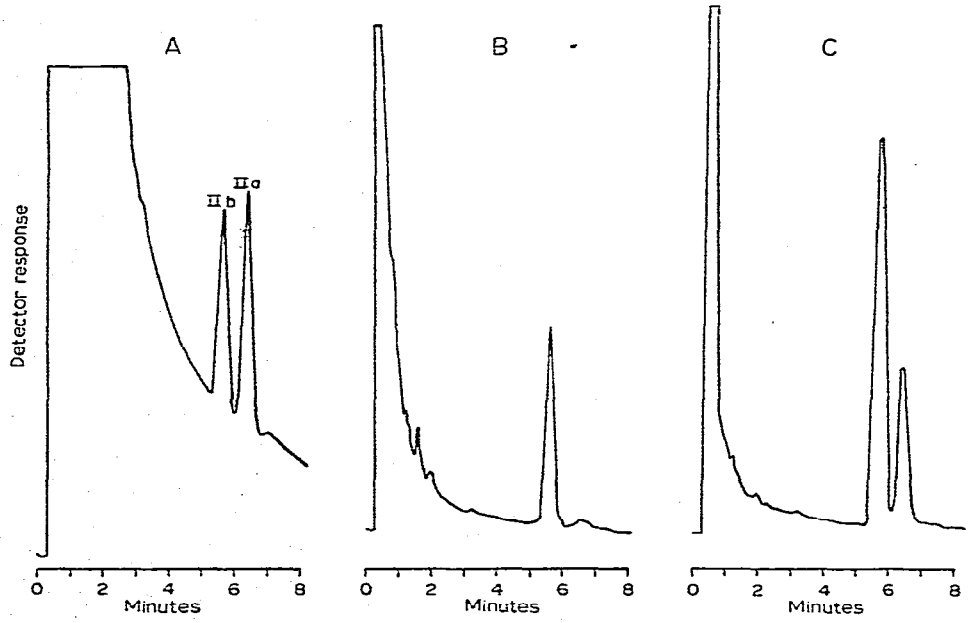


Fig. 1. Gas–liquid chromatograms of silyl derivatives of hydrolyzed urinary sample extracts separated on ITLC-SG. Column, 6 ft. × 2 mm I.D., glass; packing, 3% OV-225 on 100–120 mesh Gas-Chrom Q; column temperature, 230°; nitrogen carrier gas flow-rate, 30 ml/min. A = Guinea-pig control urine with 50 μg IIa and IIb added. B = 24-h pooled rat urine sample after 40-mg injection of II · HCl, s.c. C = 24-h pooled guinea-pig urine sample after 40-mg injection of II · HCl, s.c.

TABLE III

RELATIVE R_t ON OV-17 AND OV-225 OF DRUGS AND 6-HYDROXY METABOLITES WITH HYDROMORPHONE STRUCTURES

Relative R_t values reported are the mean of triplicate determinations. Values in brackets represent uncorrected R_t.

*Compound**	*Relative R_t on OV-17 (min)*				*Relative R_t on OV-225 (min)*			
	Underivatized compound	*Column temp. (°C)*	*Silyl derivative*	*Column temp. (°C)*	*Underivatized compound*	*Column temp. (°C)*	*Silyl derivative*	*Column temp. (°C)*
I	—**	250	1.000 (9.60)	250	—	250	1.000 (9.32)***	230
Ia	—		0.965		—		0.967	
Ib	—		0.904		—		0.834	
II	—	250	1.000 (6.18)	250	—	250	1.000 (5.90)***	230
IIa	—		0.959		—		0.958	
IIb	—		0.909				0.841	
III	—	250	1.000 (12.43)	220	—	250	1.000 (9.32)***	200
IIIa	—		0.886		—		0.868	
IIIb	—		0.873				0.803	
IV	1.000 (9.11)	250	1.000 (9.21)	230	1.000 (10.80)***	250	1.000 (12.56)	200
IVa	0.867		0.872		0.753		0.850	
IVb	0.946		0.890		0.902		0.823	
V	—	250	1.000 (9.17)***	230	—	250	1.000 (11.26)	200
Va	—		0.702				0.639	
Vb	—		0.775		—		0.692	
VI	1.000 (7.24)	250	1.000 (10.50)	230	1.000 (8.99)***	250	1.000 (8.36)	215
VIa	0.736		0.710		0.565		0.700	
VIb	0.801		0.756		0.692		0.696	

* Baseline resolution generally occurs when the difference in relative retention time between compounds is ≥ 0.085. Partial resolution occurs when the difference is ≥ 0.05.

** Indicates unsatisfactory results for GLC analysis.

*** Optimum condition for separation of the α- and β-hydroxy compounds.

lowed the α-β-parent elution pattern on both phases. Knowledge of these patterns allowed predictions of the α- and/or β-hydroxy metabolite content of sample extracts analyzed by GLC without prior separation on TLC.

Analysis of rat and guinea-pig urine samples (single 40-mg dose of II·HCl, s.c.) indicated that hydroxy metabolites are found in both species. However, the stereospecificity of formation appears to be substantially greater for the rat (negligible α/β ratio) than for the guinea-pig (α/β ratio of 0.41) as shown in Fig. 1. Unhydrolyzed urine from both species followed the same pattern. These data support the observation by Cone *et al.*[2] that Ia is a metabolite of I in the guinea-pig.

Narcotic antagonistic and/or agonistic activity of the 6-α- and 6-β-hydroxy metabolites of I–VI is generally recognized[11–13]. Knowledge of the stereospecificity of the drug metabolizing enzyme(s) involved in the reduction of the C-6 keto group of the opiates should shed new light on the possible pharmacologic interactions of these active metabolites.

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