Journal of Chromatography, 137 (1977) 323–332
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CHROM. 9963

QUANTITATIVE DETERMINATION OF NALTREXONE AND NALTREXONE PRODRUGS BY ELECTRON-CAPTURE GAS–LIQUID CHROMATOGRAPHY

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(Received January 10th, 1977)

SUMMARY

An electron-capture gas chromatographic method has been applied to the determination of eleven ester and ether derivatives of naltrexone believed to act as prodrugs. Standard analytical curves are presented for all prodrugs and quantitation is shown to be possible from 10 ng to 1.5 μg of each compound. Ester derivatives of naltrexone are hydrolyzed to naltrexone prior to analysis as the perfluoroalkyl esters. Analysis of synthetic mixtures of these with naltrexone demonstrated that quantitation by difference measurements is possible with naltrexone–derivative ratios from 4:1 to 1:10. Ether derivatives are analyzed without hydrolysis. This method is applicable to biological fluids as well as aqueous solutions.

INTRODUCTION

Naltrexone, synthesized by Blumberg *et al.*[1] in 1965, is a potent rapidly acting narcotic antagonist which is undergoing extensive investigation in both clinical trials for the prevention of narcotic addiction in man[2–5] and fundamental studies of its metabolism[6–10].

Considerable analytical methodology has been developed to measure small quantities of naltrexone and its metabolites. Verebely *et al.*[11] in 1975 reported a method for determining naltrexone (NTX) and its metabolite, beta-naltrexol in human urine based on flame ionization detection of a silylated derivative. They reported an absolute level of sensitivity of 10–20 ng/ml. Verebely *et al.*[12] in 1976 reported a method for determining NTX and beta-naltrexol using prior esterification with pentafluoropropionic anhydride (PFPA) and detection after gas chromatography with electron-capture detection (GLC–ECD). Recently, Sams and Malspeis[13] have reported the determination of NTX and its metabolites as PFPA derivatives by GLC–ECD using a more efficient base-catalyzed derivatization procedure.

NTX itself, has only a limited duration of activity (from 2 h to 2 days depending on its mode of administration) and efforts are being made to increase the duration of activity by various means. NTX in poly(lactic acid) composites[14,15], NTX in lactic–glycolic acid polymers[16], NTX in salt complexes with large organic anions and vari-

TABLE I

NAMES, ABBREVIATIONS, STRUCTURES AND FORMULAE OF NALTREXONE PRODRUGS USED IN THIS STUDY

(Structure: naltrexone skeleton with RO at position 3, HO, N—CH_2—cyclopropyl, O, =O)

Abbreviation	*Name**	*Formula*	*MW (g/mole)*	*R (TS = —C(O)—C_6H_4—C(O)NH—C_6H_4—SO_2NH—)*
NTX	Naltrexone	$C_{22}H_{22}N_1O_4$	341.4	—H
NT-Pal	NTX-3-palmitate	$C_{36}H_{53}NO_5$	579.8	$—C(O)—(CH_2)_{14}—CH_3$
NT-Stear	NTX-3-stearate	$C_{38}H_{57}NO_5$	607.9	$—C(O)—(CH_2)_{16}—CH_3$
NT-X9	NTX-3-xanthene-9-carboxylate	$C_{34}H_{31}NO_6$	549.6	xanthene-9-yl —C=O
NT-Tere-NT	NTX-3-terephthaloyl-NTX	$C_{48}H_{48}N_2O_{10}$	812.9	—C(O)—C_6H_4—C(O)—
NT-DMAT	NTX-3-terephthalotl-2,6-dimethylaniline	$C_{36}H_{36}N_2O_6$	592.7	—C(O)—C_6H_4—C(O)—NH—$C_6H_3(CH_3)_2$
NT-Sulfacet	NTX-3-terephthaloyl sulfacetamide	$C_{36}H_{35}N_3O_9S$	685.8	—TS—C(O)—CH_3
NT-Sulfadox	NTX-3-terephthaloyl sulfadoxine	$C_{40}H_{39}N_5O_{10}S$	781.8	—TS—NH—pyrimidine(OCH_3, OCH_3)
NT-Sulfadi	NTX-3-terephthaloyl sulfadimethoxine	$C_{40}H_{39}N_5O_{10}S$	781.8	—TS—NH—pyrimidine(CH_3O, OCH_3, H)
NT-MeEther	NTX-3-methyl ether	$C_{21}H_{25}NO_4$	355.4	$—CH_3$
NT-EtEther	NTX-3-ethyl ether	$C_{22}H_{27}NO_4$	369.4	$—CH_2CH_3$
NT-nButEther	NTX-3-*n*-butyl ether	$C_{24}H_{31}NO_4$	397.5	$—(CH_2)_3—CH_3$

* NTX indicates naltrexone in the name, *i.e.*, NTX-3-palmitate is naltrexone-1 3-palmitate.

ous metals[17,18], NTX derivatives which act as prodrugs[19–23], and salt complexes of NTX prodrugs with large organic anions[23], have all been used to prolong the activity of NTX. The use of NTX prodrugs to prolong the duration of the antagonist effect necessitated the development of analytical procedures for quantitating these prodrugs.

In this report, methods for determining the concentration level of eleven NTX prodrugs at the submicrogram level in aqueous solutions and biological fluids as perfluoroalkyl derivatives by GLC–ECD are presented.

EXPERIMENTAL

Reagents

Naloxone (NAL) hydrochloride and naltrexone hydrochloride were obtained from the National Institute on Drug Abuse (Rockville, Md., U.S.A.) and were converted to the free base (see Table I for structures). The NTX prodrugs, listed in Table I, were synthesized by and obtained from Drs. Bhat, Malspeis and Reuning at The Ohio State University College of Phatmacy (Columbus, Ohio, U.S.A.).

Pentaflucropropionic anhydride (PFPA) was obtained from PCR (Gainsville, Fla., U.S.A.) and used without further purification. 4-Dimethylaminopyridine (DMAP) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and purified by recrystallization from benzene. Methanol and benzene were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Standarized 1 *M* NaOH and 1 *M* HCl solutions were obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.). All other chemicals were reagent grade and used without further purification.

Glassware

All glassware was acid washed and siliconized by the method of Sams and Malspeis[13].

Stock solutions

Stock solutions of NTX and NAL (0.01 mg/ml) in methanol were prepared. No evidence of decomposition as detected by GLC–ECD was observed at 25° for three months. Stock solutions of the prodrugs (0.1 mg/ml) in methanol were prepared and used immediately. Some decomposition (*ca.* 10%) was observed after two weeks. A stock solution of 1% DMAP catalyst in benzene was prepared fresh daily.

Hydrolysis

Acid-catalyzed hydrolysis of the NTX prodrugs containing ester linkages was carried out by combining 0.5 ml of 1 *M* HCl with 1 ml of solution containing the prodrug in a siliconized screw-cap test tube. The tube was tightly capped with a Teflon®-lined cap and heated completely immersed in an oil-bath at 110° (Hallikainen Instrument, Richmond, Calif., U.S.A.). The tube was removed after 5 h and cooled. The acid was neutralized with 0.5 ml of 1 *M* NaOH.

Extraction and derivatization

All samples in aqueous solution or biological fluids were extracted into benzene and derivatized using the method of Malspeis *et al.*[9] and Sams and Malspeis[13]. Each sample was placed in a siliconized screw-capped 15 × 125-mm test tube and 0.5 g

NaCl, 2.5 ml phosphate buffer (pH 10.4, ref. 24) and 5.5 ml benzene were added. This mixture was shaken for 15 min and centrifuged to separate the layers (10 min for biological samples, 30 sec for aqueous samples). The benzene layer was transferred to another tube and evaporated to dryness in a stream of nitrogen. This was further dried at least 16 h in a vacuum dessicator. To the tube was added 25 μl PFPA and 50 μl 1% DMAP in benzene. The contents were thoroughly mixed and the tube was tightly sealed with a Teflon-lined screw cap. The tube was placed in an oil-bath at 68° to a depth of about 50 mm so the upper part of the tube acted as a reflux condensor. After 1 h, the tube was removed and placed in an ice-bath. Excess derivatizing agent was removed before chromatography by adding 5 ml of a saturated aqueous solution of sodium borate to the cooled reaction mixture. This was mixed for 3 min and centrifuged to separate the layers. Aliquots (1–3 μl) of the upper (organic) layer were used for chromatographic analysis.

Electron-capture gas–liquid chromatography

Analyses were carried out using a Hewlett-Packard (Palo Alto, Calif., U.S.A.) 5714A gas chromatograph equipped with a ^{63}Ni electron-capture detector (pulsed, variable-frequency type). Chromatographic columns were 183 cm × 2 mm I.D. coiled borosilicate glass treated with trimethylchlorosilane (5% in toluene). With the aid of a gentle vacuum and moderate vibration, columns were packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). Packed columns were conditioned at 325° for 4 h with no carrier flow and then at 275° for 16 h with carrier flow.

The carrier gas was argon–methane (95:5) at a flow-rate of 30 ml/min. Separations were carried out isothermally at 205°. The injection port was at 250° and the detector temperature was 300°.

RESULTS AND DISCUSSION

Two representative chromatograms are shown in Fig. 1. Fig. 1a is a chromatogram of derivatized NAL, NTX and NT-MeEther. Prodrugs in which ether linkages are formed with naltrexone (NT-MeEther, NT-EtEther and NT-nButEther) do not undergo hydrolysis readily and are detectable as their perfluoroalkyl derivatives under these chromatographic conditions. Fig. 1b is a chromatogram of NAL and NT-Stear after it has been hydrolyzed to NTX again detected as the perfluoroalkyl derivative. All of the prodrugs listed in Table I, other than the ether prodrugs, are hydrolyzed before derivatization and detected as NTX.

Standard analytical curves were determined for the prodrugs listed in Table I. Varying amounts of prodrug (1–20 μl of an 0.1-mg/ml methanol solution) were added to 1 ml water along with 100 ng NAL as an internal standard. These solutions were hydrolyzed, extracted, derivatized and chromatographed. The peak heights for the internal standard (NAL) and sample (prodrug) were measured and the peak height ratio (PHR) of the sample to standard calculated. The slopes and intercepts of the analytical curves were calculated using a linear least squares regression on eqn. 1:

$$\text{ng of prodrug} = \text{slope} \cdot \text{PHR} + \text{intercept} \quad (1)$$

Several analytical curves are shown in Fig. 2 and values for the slopes, inter-

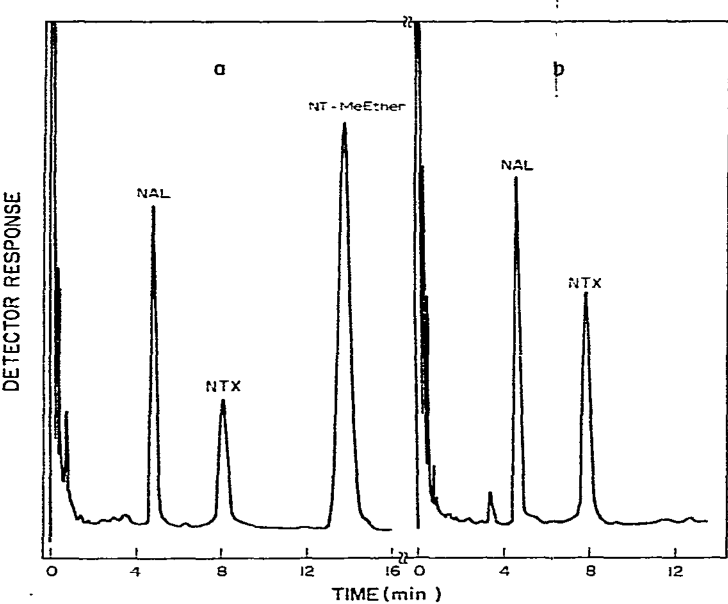

Fig. 1. Chromatograms obtained on an 8 ft. × 2 mm I.D. column packed with 3% OV-17 on Gas-Chrom Q. Column temperature, 205°; carrier gas, 5% methane in argon at a flow-rate of 30 ml/min. (a) 100 ng NAL, 60 ng NTX and 630 ng NT-MeEther derivatized with PFPA without prior hydrolysis. (b) 100 ng NAL and 180 ng NT-Stear derivatized with PFPA after hydrolysis.

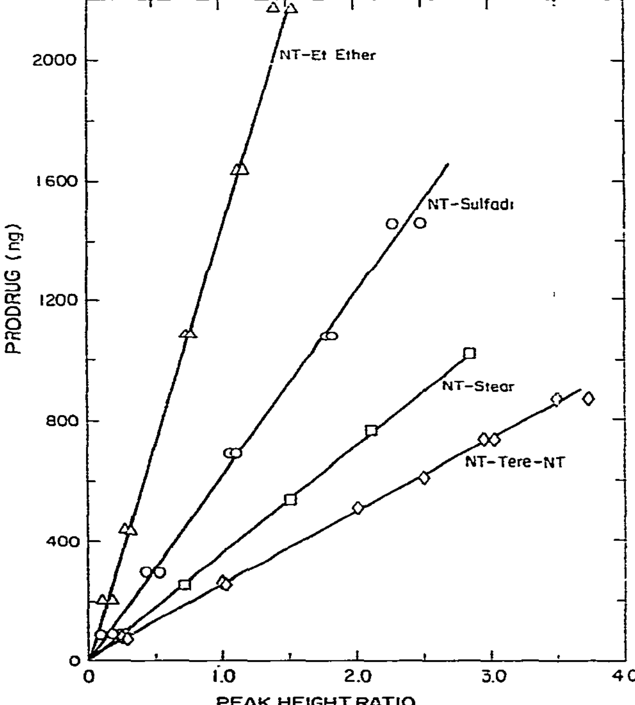

Fig. 2. Representative standard analytical curves for four of the prodrugs listed in Table II.

TABLE II

SUMMARY OF ANALYTICAL CURVE PARAMETERS FOR THE NALTREXONE PRODRUGS

Numbers in parentheses are standard deviotions. n = Number of samples used. Analytical curve for NTX itself determined the same experiment as the prodrug slope. The average standard deviation of the slope of NTX analytical curves is 0.46 ng; linearized intercept standard deviation is 15.2 ng. Slope ratio as determined by eqn. 2.

Prodrug	*Slope (ng)*	*Intercept (ng)*	*Corr. coeff. (%)*	*n*	*NTX slope*	*Intercept*		*Slope ratio*
						NTX	*NTX + prodrug*	
NT-Pal	266.1 (4.8)	3.5 (6.2)	99.95	10	151.8	− 9.2	− 8.5	1.03
NT-Stear	355.7 (6.0)	− 2.7 (7.2)	99.97	9	197.5	− 9.4	− 5.7	1.02
NT-X9	288.1 (7.5)	−20.7 (18.6)	99.8	10	172.3	+ 2.0	− 3.5	1.03
NT-Tere-NT	226.1 (10.9)	28.9 (22.9)	99.2	11	180.2	− 0.5	−20.3	1.05
NT-DMAT	446.8 (13.1)	10.7 (18.0)	99.6	8	210.7	−12.7	4.5	1.22
NT-Sulfacet	493.1 (10.4)	− 5.3 (4.6)	99.8	8	207.8	1.2	11.3	1.18
NF-Sulfadox	413.6 (18.8)	−18.7 (10.3)	99.3	10	188.2	− 7.6	−17.4	0.96
NT-Sulfadi	601.8 (17.1)	3.75 (11.9)	99.7	8	210.5	− 3.3	−21.7	1.25
NT-MeEther	518.5 (13.9)	22.0 (32.2)	99.8	10	—	—	—	—
NT-EtEther	2182.6 (18.8)	4.5 (15.7)	99.9	10	—	—	—	—
NT-nButEther	2931.4 (36.2)	−12.5 (9.8)	99.1	8	—	—	—	—

cepts, standard deviations and correlation coefficients are listed in Table II. The prodrugs which are ether derivatives have retention times of 13.8 min for NR-MeEther, 15.6 min for NT-EtEther and 27.7 min for NT-nButEther in comparison with the NAL retention time of 4.7 min and NTX of 8.1 min. The standard deviation of all slopes is less than 5% and all intercepts are within a 95% confidence interval of zero. A standard analytical curve for NTX itself was determined at the same time as that for each hydrolyzable prodrug. There is a significant variation in these values. Variations in room temperature during the extraction procedure, in column material from different lots, in the reproducibility of column characteristisc due to both packing technique and column, age and other column parameters lead to the variability seen in the NTX analytical curve. This curve apparently must be determined along with every run. The average standard deviation for the NTX analytical curve is 4.6 ng for 81 individual determinations.

The hydrolysis of the prodrugs with ester linkages should follow eqn. 2:

$$\text{prodrug} + H_2O \leftrightarrows \text{NTX} + \text{acid} \tag{2}$$

One molecule of NTX is formed for every molecule of the prodrug present if hydrolysis is complete. Therefore, the slope of the analytical curve for a particular prodrug should be proportional to the slope ratio for NTX determined simultaneously, with the proportionality constant being the ratio of the molecular weight (MW) of NTX to the particular prodrug. This slope ratio can be calculated according to eqn. 3:

$$\text{slope ratio} = \frac{\text{slope (prodrug)} \cdot \text{MW (NTX)}}{\text{slope (NTX)} \cdot \text{MW (prodrug)}} \tag{3}$$

The slope ratios for the prodrugs having ester linkages are listed in Table II. If hydrolysis is complete the slope ratio should equal 1.

Of the prodrugs having an ester linkage to NTX, several have slope ratios greater than 1. The cause for this deviation may be incomplete hydrolysis. The rates of hydrolysis of several of the NTX prodrugs were measured under conditions outlined earlier for hydrolysis of the esters. The amount of NTX formed from a prodrug was measured at various time intervals. The hydrolysis was found to follow first order kinetics. The hydrolysis rate constant is calculated using eqn. 4:

$$\ln(\mathrm{NTX_f} - \mathrm{NTX_t}) = k_{\mathrm{obs.}} \cdot t + \ln \mathrm{NTX_f} \quad (4)$$

where NTX_f is the final and NTX_t the concentration at time t, respectively, and $k_{obs.}$ is the pseudo first-order rate constant. The values for three prodrugs are listed in Table III. Hydrolysis rates were measured for prodrugs having the three main types of ester linkages present in the prodrugs: an aliphatic ester (NT-Stear), a linkage to a xanthene group (NT-X9) and an aromatic ester (NT-Sulfadi). NT-Stear and NT-X9 are fast enough to assure over 99% hydrolysis under these conditions. However, the NT-Sulfadi hydrolysis rate is much slower as would be expected for an aromatic ester linkage[25] and only 87% of this prodrug has hydrolysed in 5 h. Therefore, the slope ratio would be greater than 1 as is seen for several of the prodrugs listed in Table II.

TABLE III

KINETIC PARAMETERS OF HYDROLYSIS OF PRODRUGS AND THE DECOMPOSITION OF NALTREXONE AND NALOXONE UNDER ASSAY CONDITIONS

Numbers in parentheses are standard deviations.

Sample	*$k_{obs.}$ (h^{-1})*	*Intercept*	*Corr. coeff. (%)*
NT-Stear	1.0 (0.1)	5.34 (0.25)	93.7
NT-X9	0.94 (0.08)	6.04 (0.19)	93.2
NT-Sulfadi	0.40 (0.02)	5.54 (0.04)	96.4
NAL	0.0699 (0.008)	4.57 (0.02)	91.8
NTX	0.0691 (0.008)	4.58 (0.02)	91.8

The hydrolysis reaction could be driven further toward completion by either increasing the temperature of the reaction or by carrying on the hydrolysis longer. However, the decomposition of NAL and NTX prohibits this. NAL and NTX are observed to decompose slowly under the conditions of the hydrolysis. The rates of decomposition of NAL and NTX were measured and the first-order rate constants are found to be essentially the same (Table III). The loss of NAL and NTX under hydrolysis conditions causes a 30% drop in sensitivity. If the hydrolysis were continued for a longer period of time or at a higher temperature, the sensitivity would be decreased further. Even though there is incomplete hydrolysis for some of the prodrugs under the condition used, this does not effect the ability to measure these prodrugs. The excellent linearity of the analytical curves permits analysis even when hydrolysis is incomplete and using a longer hydrolysis time or higher temperatures would only tend to decrease sensitivity.

The presence of unhydrolyzed prodrug does not interfere with the measurement of NTX. Analytical curves for NTX in the presence and absence of 1.5 μg of prodrug were within 1% of each other and the intercepts, listed in Table II, also do not vary. If this large amount of prodrug were interfering with the analysis NTX, a large negative intercept would be expected in analytical curves where prodrug was present.

A series of synthetic mixtures was made up to determine the accuracy in measuring NTX and a hydrolyzable prodrug in solution together. Various known amounts of NTX (30–400 ng) and NT-Stear (150 ng to 1.5 μg) were added to 1 ml of water. Four identical samples were made up for each of six combinations of NTX and NT-Stear. Two samples were then extracted, derivatized, and chromatographed and the peak height ratios for the hydrolized [PHR (H^+)] and unhydrolyzed [PHR (noH^+)] samples determined. The amounts of NTX and NT-Stear in each set of samples were determined using eqns. 5 and 6:

$$\text{ng NTX} = \text{slope (NTX)} \cdot \text{PHR (noH}^+\text{)} + \text{intercept (NTX)} \tag{5}$$

$$\text{ng NT-Stear} = \text{slope (NT-Stear)} \cdot \text{PHR (H}^+\text{)} + \text{intercept (NT-Stear)} \tag{6}$$

The slopes and intercepts of NTX and NT-Stear in eqns. 5 and 6 were determined at the same time. The results found for six sample mixtures (average of two determinations) are listed in Table IV. The amounts found are in good agreement with the amounts added. The large percent deviations are only found for samples where a small amount of NTX is present with a large amount of prodrug. As little as 0.5% hydrolysis of NT-Stear could cause the 12–17% deviation seen in the NTX values at the large ratios of prodrug to drug.

Analytical curves were determined from the prodrugs in 1-ml plasma and urine samples. Samples were extracted with benzene before hydrolysis in order to remove many of the other constituents of plasma and urine which could cause NTX or NAL to decompose more rapidly under the hydrolysis conditions. The resultant

TABLE IV

ANALYSIS OF MIXTURES OF NALTREXONE AND NALTREXONE-3-STEARATE OF KNOWN COMPOSITION

The amount of NTX and NTX-Stear was calculated using eqns. 5 and 6, and slope (NTX) = 181.1, intercept (NTX) = 3.7; slope (NT-Stear) = 363.8, intercept (NT-Stear) = 2.8; 100 ng NAL was used as an internal standard.

Samples were added using a 10-μl Hamilton syringe and stock solutions of 10 ng/μl NTX and 150 ng/μl NT-Stear.

Deviation (%) = (amount found/amount added) × 100%.

Sample	*Naltrexone*			*Naltrexone-3-stearate*		
	Added (ng)	*Found (ng)*	*Deviation (%)*	*Added (ng)*	*Found (ng)*	*Deviation (%)*
1	30	30.3	+ 1.0	300	319	+6.4
2	100	103.0	+ 3.0	900	870	−3.3
3	30	35.1	+17.0	1500	1469	−2.1
4	60	67.3	+12.1	1500	1556	+3.8
5	200	198.3	− 0.9	150	146	−2.5
6	400	395.9	− 1.2	750	796	+6.2

TABLE V

DETERMINATION OF ANALYTICAL CURVES OF VARIOUS PRODRUGS FROM DOG PLASMA AND HUMAN URINE

Numbers in parentheses are standard deviations. R = correlation coefficient (%).

Sample	*Plasma*			*Urine*			*Aqueous*		
	Slope (ng)	*Intercept (ng)*	*R (%)*	*Slope (ng)*	*Intercept (ng)*	*R (%)*	*Slope (ng)*	*Intercept (ng)*	*R (%)*
NTX	181.3 (4.1)	− 7.3 (11.5)	99.95	179.4 (0.8)	0.7 (3.4)	99.99	185.2 (2.1)	− 2.25	99.98
NT-Stear	524.3 (20.1)	−18.1 (1.52)	99.91	383.9 (7.4)	−10.1 (22)	99.03	355.7 (6.0)	− 2.7 (7.2)	99.97
NT-Tere-NT	266.4 (5.4)	−25.5 (20.5)	99.7	274.8 (6.4)	−26.7 (25)	99.8	248.2 (6.9)	− 7.9 (27)	99.5
NT-MeEther	490.2 (13.4)	43.8 (56.6)	99.74	552.4 (12.1)	− 3.3 (74.1)	99.83	518.5 (14)	22 (32)	99.71

benzene solution was evaporated to dryness and 1 ml water and 0.5 ml 1 *M* HCl were added and the sample hydrolyzed. After hydrolysis the samples were again extracted and treated as previously described. The resultant slopes, intercepts and correlation coefficients for NTX and three of the NTX prodrugs are listed in Table V along with their standard deviation. These analytical curves show good linearity and therefore the ability to quantitate the NTX prodrugs when they are present in either plasma or urine. The increased slope (lower sensitivity) for some of the prodrugs when extracted from urine and plasma may be due to some factor such as binding between the prodrug and proteins.

ACKNOWLEDGEMENTS

This work was supported by the National Institute of Drug Abuse contract 1-MH-3-0182(ND) and grant 1 RO1 DA 00473-01.

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