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ANALYSIS OF N-*n*-PROPYLNORAPOMORPHINE IN PLASMA AND TISSUE BY CAPILLARY GAS CHROMATOGRAPHY—ELECTRON-CAPTURE DETECTION

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SUMMARY

Capillary gas chromatography combined with electron-capture detection (GC—ECD) was applied to the detection and quantitation of N-*n*-propylnorapomorphine (NPA) and related compounds in serum and tissue using trifluoroacetyl (TFA) derivatives. The detection limits for NPA using GC—ECD of TFA derivative extend into the subpicogram level, but quantitation in serum was limited to levels of 100 ng/ml due to matrix interferences. The method was applied to the analysis of NPA in rat serum after administration of a moderate dose of the drug and was applied to the detection of NPA in rat brain after the peripheral administration of (—)10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA). These results support previous proposals that MDO-NPA is a prodrug of NPA, which acts at cerebral dopamine-receptors.

INTRODUCTION

Apomorphine (APO) and N-*n*-propylnorapomorphine (NPA) have potent and selective actions at central and other dopamine (DA) receptors [1]. They have been used clinically especially in neurological and psychiatric disorders [2—5] although their clinical use has been limited by their poor oral bioavailability and short duration of action [1,6]. A prodrug of NPA, (—)10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA), has recently been described [7,8] as a unique, orally effective and long-acting apomorphine derivative that exerts activity at DA receptors in the brain.

In view of the clinical significance of APO, NPA and MDO-NPA (structures in Fig. 1) the detection and quantitation of these drugs in biological fluids are of

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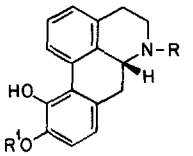

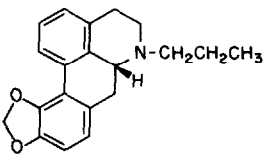

(-) Apomorphine (APO), R = CH_3, R^1 = H

(-) N-n-propylnorapomorphine (NPA), R = $CH_2CH_2CH_3$, R^1 = H

(-) Apocodeine, R = R^1 = CH_3

(-) N-n-propylnorapocodeine (10-O-Me-NPA), R = $CH_2CH_2CH_3$, R^1 = CH_3

(-)10,11-Methylenedioxy-N-n-propylnoraporphine (MDO-NPA),

Fig. 1. Structures of apomorphine (APO), N-*n*-propylnorapomorphine (NPA), apocodeine N-*n*-propylnorapocodeine (10-O-Me-NPA), and 10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA).

interest. In order to establish more closely the correlation between tissue levels of these drugs and their pharmacological effects in vivo, several analytical methods for the assay of APO and NPA have been investigated. Techniques such as thin-layer chromatographic fluorescence quenching [9], spectrophotometry [10], paper chromatography [11], fluorometry [12], gas chromatography using flame ionization or thermal conductivity detectors [13,14] and high-performance liquid chromatography using UV or electrochemical detectors [15–19] have reported sensitivities ranging from 5 ng/ml to a few hundred ng/ml of biological fluid without the need for chemical derivatization. In a recent publication, Watanabe et al. [20] have reported detection limits of 30 ng/ml for APO using trimethylsilyl derivatives and gas chromatography—mass spectrometry (GC—MS) in the single ion monitoring mode. A less sensitive radioenzymatic assay of APO has also been developed [21].

While many of the techniques summarized above offer specific advantages, a number of drawbacks may also be noted. For example, most of the GC work was conducted with conventional packed columns and flame ionization detectors. Under these conditions detection limits of, at best, 1 ng have been reported. On the other hand, even though GC—MS single ion monitoring is capable of detection at the picogram level, the technique is costly and not generally accessible. In view of the increasing clinical significance of NPA and MDO-NPA we investigated the utility of fused silica capillary GC—electron-capture detection (GC—ECD) for the analysis of these compounds in plasma and urine. While packed column GC—ECD has been used previously for the determination of APO in equine plasma [22], it was reasoned that the fused silica capillary GC would provide us with superior chromatographic resolution for identification of the parent drugs or their metabolites with a much higher degree of confidence based on retention times. Moreover, it was anticipated that the well established sensitivity of the electron-capture detector should permit detection and quantitation at the picogram level or lower. It was felt that use of the relatively simple capillary GC—ECD combination should make available a simple and inexpensive method for assaying these compounds in a clinical laboratory. Our studies reported here focused on the analysis of NPA and related compounds

in plasma and tissues, after the periphenal administration of both NPA and MDO-NPA.

Our approach was based on the use of a three-step extraction procedure for isolation of the alkaloids, formation of trifluoracetyl derivatives to enhance the electron affinity and volatility of the solutes and finally analysis by capillary GC–ECD. This methodology was applied to in vivo studies in rats. The enzymatic transformation of MDO-NPA to NPA in rat brain was confirmed by the detection and quantitation of NPA in the brain tissue, following administration of the parent drug. The results of this study are reported below.

EXPERIMENTAL

Materials

N-*n*-propylnorapomorphine (NPA), apomorphine (APO), 10-O-methyl-N-*n*-propylnorapomorphine (10-O-Me-NPA) and 10,11-methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA) were synthesized in these laboratories as their hydrochloride or hydrobromide salts. The MDO-NPA was analyzed for its NPA content by GC–ECD. The NPA contamination of this material was found to be 0.01%. Radioactive [^{3}H] NPA was provided by New England Nuclear, Boston, MA, U.S.A. (0.25 μg, specific activity 60.0 Ci/mmole). All organic solvents were chromatography grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). The other reagents were of analytical research grade. The glassware was treated with a hot solution of 5% (v/v) hexamethyldisilazane in toluene for 15 min to reduce surface adsorption.

Preparation of standards

A standard stock solution of N-*n*-propylnorapomorphine-HCl (NPA) (equivalent to 1 mg free amine per 10 ml) was made up in methanol and stored under refrigeration for no more than one week. The working standard was diluted to a final free amine concentration of 1 μg/ml. Triplicate standards containing increasing concentrations of NPA·HCl were prepared by measuring 100, 200, 400, 600, 800 and 1000 μl of working standard solution into screw-cap vials. The solvent was removed under nitrogen and the residues derivatized as described below.

Standard solutions of the other two compounds, APO and 10-O-Me-NPA, and the mixture of the three were prepared in a similar fashion.

Extraction

Biological samples of the three-component mixture containing 1 μg or 10 μg of each solute per ml of plasma were prepared by spiking 1 ml of a rat plasma with the appropriate amount of the drug. The plasma mixture (1 ml) was mixed with sodium triphosphate buffer (1.0 ml, pH 7.4). This mixture was fortified with 1 mg of dithiothreitol to prevent oxidation of the amines [14] and extracted by shaking with three 5-ml portions of ethyl acetate. The dithiothreitol was included in every step of the extraction process. To minimize interference with the GC analysis from any coextracted plasma constituents, the ethyl acetate extracts were further purified by a back extraction as follows: 5 ml of 0.2 *M* aqueous hydrochloric acid was added to the combined ethyl acetate extracts and the amine solutes extracted into the aqueous layer as their hydro-

chloride salts. The aqueous layer was washed once with 5 ml *n*-hexane to remove any lipophilic impurities and the pH was adjusted to near 7.4 by addition of 1 *M* sodium hydroxide and 2 ml of pH 7.4 buffer. The free amines were re-extracted into three 5-ml portions of ethyl acetate. After each extraction the tubes were centrifuged for 10 min to define better the separation of the two phases. A comparison of the GC profiles of a plasma extract before and after back extraction will be shown later in Fig. 4, indicating the advantage obtained by introducing a second purification step.

The efficiency of the extraction procedure was determined using tritium labeled NPA (specific activity 60 Ci/mmole) [23]. Plasma samples (1.0 ml) were each spiked with 10 μg, 1.0 μg and 0.1 μg of NPA. To each 1.0-ml plasma sample 0.12 ng of radioactive NPA was added. The results, indicating the percent recovery in each of the three extraction steps, are summarized in Table I. The average of six determinations are given along with the percent standard deviation in each set of six. It is apparent that, while the bulk of the NPA can be recovered following the first extraction, at least a second extraction step is necessary to assure near full recovery. It should be noted, however, that even after a single extraction, a very large fraction of the lipophilic constituents of the plasma was co-extracted. Back-extraction, as described above, is therefore necessary in order to obtain a sample relatively free of coeluting interferences for the GC—ECD analysis of the NPA. As a result of these additional cleanup steps, final sample recovery corresponded to approximately 75% of the original sample.

TABLE I

EXTRACTION EFFICIENCY OF NPA FROM PLASMA INTO ETHYL ACETATE

For details of analysis see Experimental section. Values are the average of six determinations; values in parentheses refer to percent standard deviation in each group of six determinations.

NPA (μg/ml plasma)	Recovery* (%)			
	Extraction 1	Extraction 2	Extraction 3	Total
10.0	94.0 (5.9)	8.7 (0.4)	1.4 (0.2)	104.3 (6.0)
1.0	94.0 (4.4)	8.6 (1.2)	1.4 (0.1)	103.9 (4.6)
0.1	90.5 (7.2)	7.7 (0.5)	1.3 (0.1)	99.4 (7.6)

*Total recovery for the group of all 18 samples correspond to 102.5% with a standard deviation of 6.3%.

Preparation of derivatives

TFA derivatives of NPA and other related compounds were prepared according to procedures described in a previous publication [24]. The ethyl acetate extracts of the free amines, or the combined ethyl acetate plasma extracts, were evaporated under a nitrogen stream. To the residue in each vial, test or standard, was added excess of redistilled trifluoroacetic anhydride (TFAA). The vials were tightly capped with PTFE-lined screw-caps, placed in a vacuum dessicator and exposed to an infra-red heating lamp. The reaction temperature was maintained at 60—70°C for 60 min. This procedure allowed only minimal exposure to atmospheric moisture which may cause decomposi-

tion of the derivatives. After completion of the reaction, the excess reagent was removed under vacuum. The residue of the derivatized sample in each vial was then reconstituted in 1 ml toluene containing 1% TFAA which was added to prevent hydrolysis of the derivative. Volumes of 1 μl were injected into the gas chromatograph.

Gas chromatography

A Varian Model 3700 gas chromatograph equipped with both a flame ionization detector (FID) and a ^{63}Ni pulsed ECD and a Varian Model 9176 1-mV recorder were used. A Varian CDS 111 chromatography data system was employed for measurement of peak retention times and peak areas. The injector port was maintained at 270° C and the detector at 320° C. A fused-silica column (15 m × 0.025 mm I.D.) coated with SE 54 (J. & W. Scientific) was used. The initial column temperature was 170° C for 1 min, then programmed to a final temperature of 285°C at a rate of 6°C/min. Ultra high purity nitrogen was used as the carrier gas at a flow-rate of 5.0 ml/min. Nitrogen with a flow-rate of 30 ml/min was used as the make-up gas.

RESULTS AND DISCUSSIONS

Our evaluation of the practical utility of fused silica capillary GC—ECD for the analysis of NPA using TFA derivatives was conducted in three stages. For the first stage, standard samples of pure NPA, APO, and 10-O-Me-NPA were analyzed. This was followed by an examination of plasma and urine samples spiked with NPA. Finally, the methodology developed in the first two stages was applied to the analysis of a number of biologically derived samples.

Analysis of pure samples by GC—ECD

This portion of the study was necessary to establish a basis for the analysis of NPA by GC—ECD, specifically the detection limits for its trifluoroacetyl (TFA) derivatives and criteria for its quantitation. Construction of a standard curve for NPA, indicated a linear dynamic range covering more than three orders of magnitude (5 to 10,000 pg). The capability of the technique for trace level detection was further demonstrated by the fact that detection limits of 0.20 pg were determined for the TFA derivative of NPA. A signal-to-noise ratio of ca. 5:1 was obtained for a standard sample of 0.20 pg injected into the gas chromatograph. It should be emphasized, however, that to ensure reproducibility of the results at these levels, daily replacement of the glass-lined insert, used in the injection port of the capillary gas chromatograph, is necessary.

A chromatogram showing the separation of the three components of a standard mixture (20 pg injected of each) of NPA, APO and 10-O-Me-NPA is given in Fig. 2.

The 10-O-Me-NPA was included in the chromatogram because the latter compound is a potential metabolite of NPA, and it was important to compare its chromatographic retention with that of NPA.

Analysis of spiked serum

Serum samples spiked with standard quantities of NPA, APO and 10-O-Me-

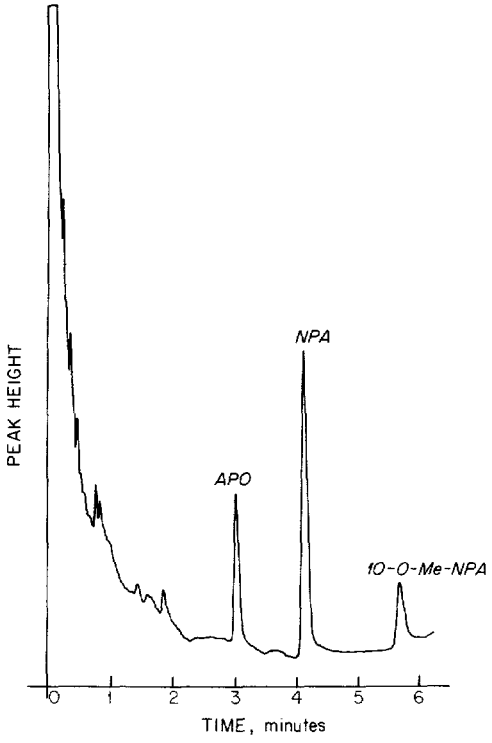

Fig. 2. Chromatogram of TFA derivatives of a standard mixture (20 pg each) of APO, NPA, and 10-O-Me-NPA. Column: 15 m fused-silica capillary, SE-54, 170°C (1 min), programmed to 285° C (6° C/min).

NPA were examined in order to establish the extraction conditions necessary for effective analysis of these compounds by capillary GC—ECD. The results obtained from a serum sample spiked with 1 μg of each compound per ml of serum are summarized in Fig. 3. A chromatographic profile of the ethyl acetate

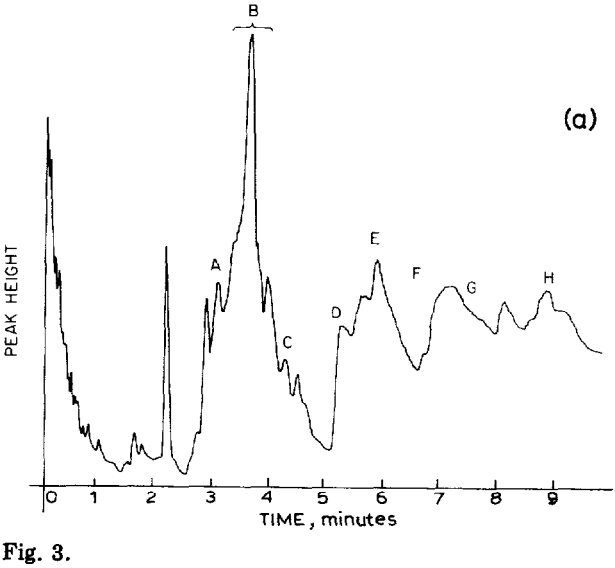

Fig. 3.

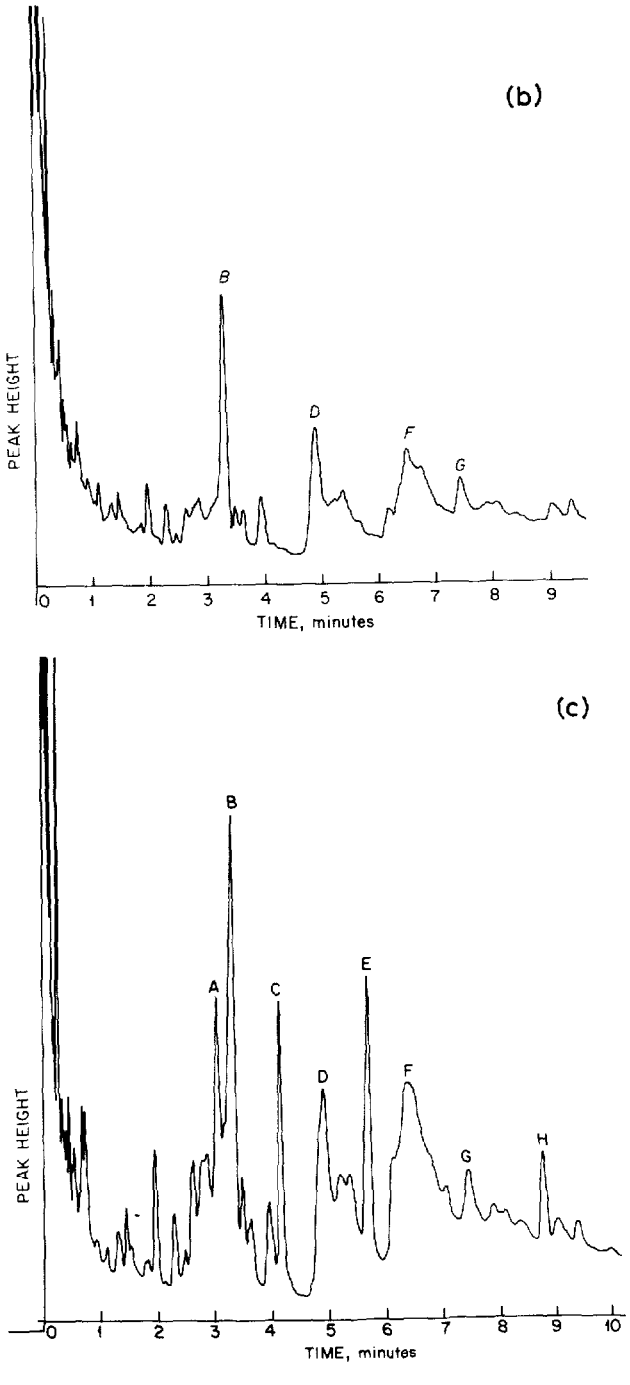

Fig. 3. GC—ECD recordings of derivatized serum extracts spiked with 1 μg/ml each of APO (A), NPA (C) and 10-O-Me-NPA (E). GC conditions as in Fig. 2. (a) spiked rat serum without back extraction; (b) serum control with back extraction; (c) spiked serum with back extraction.

extract following trifluoroacetylation is shown in Fig. 3a. Peaks A, C, and E correspond to the retention times of APO, NPA and 10-O-Me-NPA respectively. Clearly, the contributions from the serum prohibit quantitation of NPA under these conditions. This problem, however, was rectified by further clean-up of the sample via back extraction (see Experimental section). Fig. 3b shows the GC profile of the serum (control) sample after back extraction and this is compared with the corresponding profile of the spiked sample in Fig. 3c. The peaks of interest (A, C, and E) are now sharply defined permitting facile determination of the respective compounds. This is particularly the case for NPA, which elutes in a region relatively free of interferences and is thus most suitable for quantitation.

According to the radioactivity measurements discussed in the Experimental section, the back extraction process results in the retrieval of approximately 75% of the NPA added to rat serum. This value was also confirmed by the GC experiments and comparison of the NPA signal in Fig. 3c to that in the standard curve.

These results, therefore, confirmed the compatibility of the extraction conditions discussed in the Experimental section with the analysis of NPA in serum using capillary GC—ECD. Some applications of these procedures in vivo and in vitro are discussed in the following section.

Biological experiments

Two different types of in vivo applications were considered in this study in order to establish the general applicability of the method described above. The first sample (Fig. 4a) shows the chromatogram resulting from a serum sample taken 30 min after intraperitoneal administration of the drug [0.84 mg NPA; (5 mg/kg)]. Comparison with a control sample (Fig. 4b) shows good definition of the peak (A) corresponding to the retention time of NPA. The signal corresponds to 6.2 ng of NPA or 0.41 μg of NPA per ml serum after correcting for the 75% recovery in extraction. A second peak (B) in the chromatogram of Fig. 4a is presumably an unidentified metabolite of NPA. Studies using GC—MS are currently in progress to determine the nature of this and other possible metabolites.

In the second example we applied capillary GC—ECD to determine whether enzymatic conversion of MDO-NPA to NPA in the body may be responsible for the biological activity of that compound. Brain tissues from rats administered 10.5 mg of MDO-NPA per kg intraperitoneally were homogenized, extracted and derivatized as previously described. Chromatograms of extracts resulting from the treatment of control sample and samples after treatment with MDO-NPA are shown in Fig. 5. The signal arising from the NPA peak corresponds to 1.1 ng or 21 ng/g of brain tissue after correcting for the 75% recovery in extraction. The results are, in general, in agreement with those of Sperk et al. [25] who independently measured NPA levels ranging from 10—20 ng NPA per g of brain tissue for similar rat samples using high-performance liquid chromatography—electrochemical detection.

In conclusion, the data presented in this paper show that capillary GC combined with ECD provides a reliable technique for the detection and quantitation of NPA and related compounds in serum and tissue using trifluoroacetyl deriva-

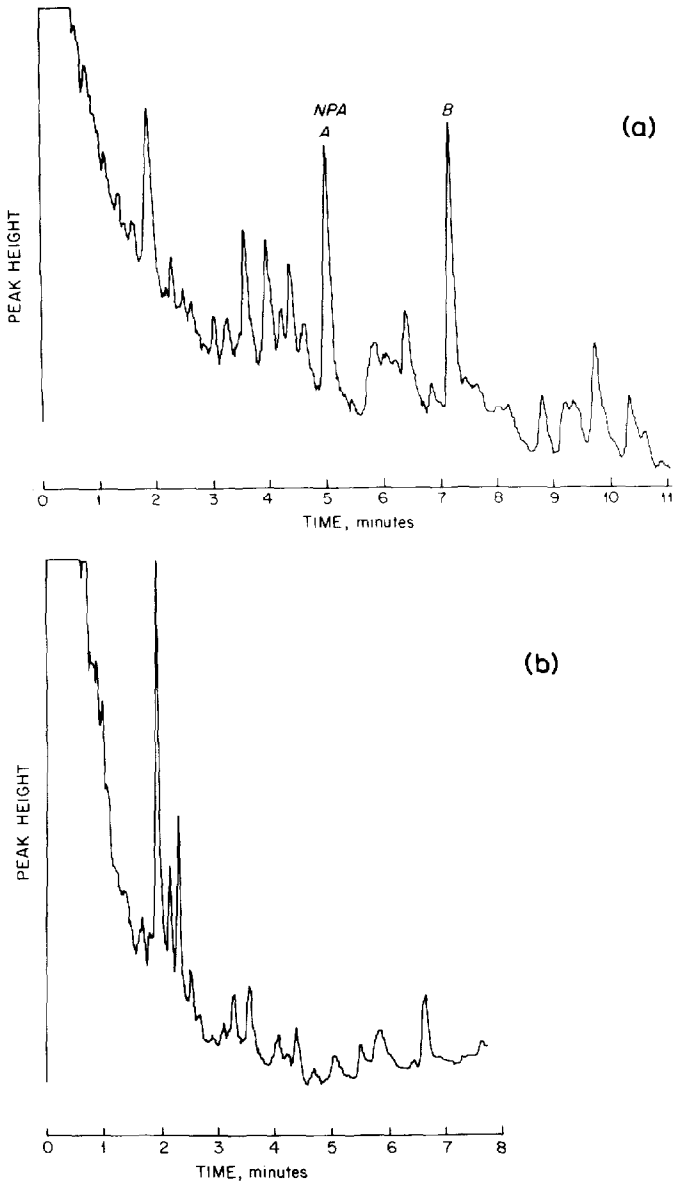

Fig. 4. GC—ECD recording of derivatized serum extract from (a) in vivo sample of rat administered 5 mg/kg NPA; (b) control sample. GC conditions as in Fig. 2.

tives. The use of a back extraction step was an effective means for cleanup of the sample prior to derivatization and GC analysis. The present results further support previous proposals that the activity of MDO-NPA in vivo may be related to its function as a prodrug to liberate NPA [7,8,25].

The detection limits for NPA using the capillary GC—ECD approach have been shown to extend into the subpicogram level while quantitation was routinely conducted to levels lower than 5 pg of sample injected into the gas chromatograph. While on an absolute basis these values compare favorably with

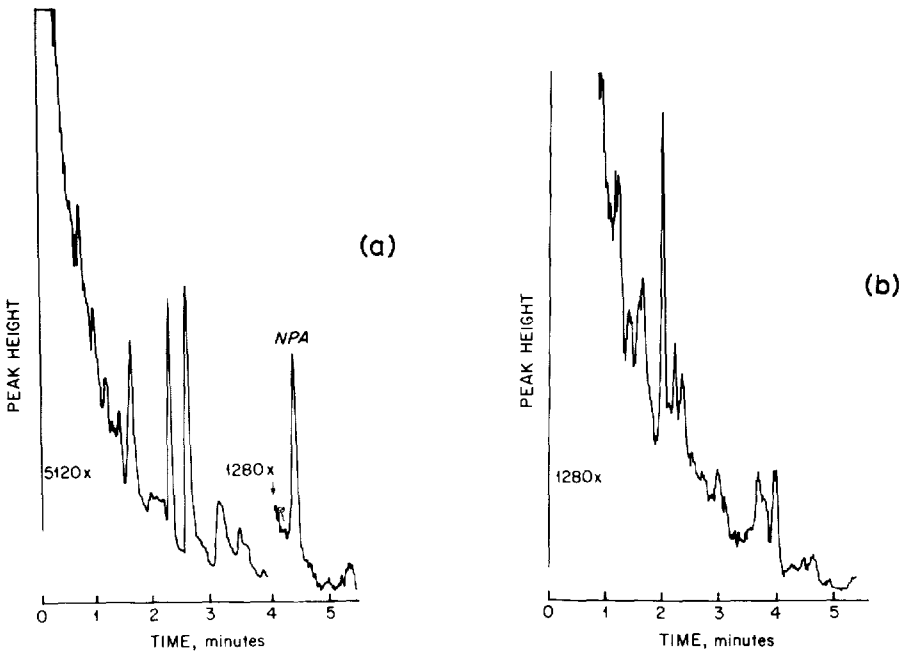

Fig. 5. GC—ECD recordings of derivatized brain tissue extracts from (a) in vivo sample of rat administered MDO-NPA (10.5 mg/kg); (b) control sample. GC conditions as in Fig. 2.

radioenzymatic (REA) or radioreceptor (RRA) assays where detection limits of 750 pg and 50 pg, respectively, are reported [6], the requirements for sample clean-up are considerably higher for the GC—ECD method. On the basis of our present data, it is estimated that the detection limits possible with the methodology developed here are, at best, in the range of 100 ng/ml of serum due to matrix interferences. This compares much less favorably to the corresponding detection limits of RRA (0.150 ng/ml) and REA (5 ng/ml). On the other hand, the GC method is not necessarily limited to the analysis of a given compound (APO or NPA), but can be applied to other constituents, such as metabolites, containing at least one derivatizable hydroxy group. Moreover, further improvements in the detection limits are anticipated via the use of more selective chromatographic detectors (e.g. mass spectrometric). Studies to that effect are currently in progress.

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