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DETERMINATION OF N-*n*-PROPYLNORAPOMORPHINE IN SERUM AND BRAIN TISSUE BY GAS CHROMATOGRAPHY–NEGATIVE ION CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A method for the determination of the neuroactive compound N-*n*-propylnorapomorphine (NPA) in biological tissues is described. Isolation of NPA from serum or brain tissue was achieved via liquid–liquid extraction from phosphate-buffered tissue extract (0.25 *M*, pH 7.2) into ethyl acetate. The NPA, along with a [$^2H_7$]NPA analogue serving as internal standard, was converted to the corresponding bis(trifluoroacetyl) ester by treatment with excess trifluoroacetic anhydride at 75°C. The electrophoric derivatives were analyzed by fused-silica capillary gas chromatography–mass spectrometry in the negative ion chemical ionization mode. Selected ion monitoring of the $[M-CF_3CO]^-$ ions of derivatized NPA (*m/z* 390) and internal standard [$^2H_7$]NPA (*m/z* 397) permitted the quantitation of NPA in serum and brain samples obtained from rats treated with either free NPA or the prodrug methylenedioxy-NPA (MDO-NPA). Calibration was conducted down to a practical limit of assay sensitivity, at 0.50 ng NPA per ml of serum and 0.50 ng NPA per g of brain. The relative standard deviation for replicate serum samples spiked at 20 ng/ml was 4.2% ($n=5$) and for brain samples at 10 ng/g, it was 3.6%. This method revealed differences in the free NPA brain/serum ratios in rats treated separately with the stereoisomers *R*-(−)-MDO-NPA and *S*-(+)-MDO-NPA.

INTRODUCTION

N-*n*-Propylnorapomorphine (NPA, 1, Table I) and apomorphine (APO, 2) are members of the aporphine class of isoquinoline-based alkaloids[1–3]. They have been

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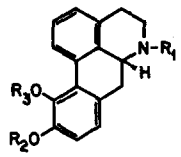
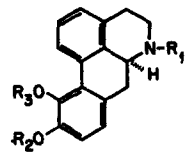
TABLE I

STRUCTURES OF APORPHINE DERIVATIVES



1a-7a, R-(-)-



1b-7b, S-(+)-

Compound	R_1	R_2	R_3
NPA (1a and 1b)	n-C_3H_7	H	H
APO (2a and 2b)	CH_3	H	H
MDO-NPA (3a and 3b)	n-C_3H_7	R_2, R_3 =	CH_2
$(TFA)_2$-NPA (4a and 4b)	n-C_3H_7	CF_3CO	CF_3CO
[$^2H_7$]NPA (5a)	n-$C_3{}^2H_7$	H	H
[$^2H_7$]$(TFA)_2$-NPA (6a)	n-$C_3{}^2H_7$	CF_3CO	CF_3CO
10,11-DME-NPA (7a and 7b)	n-C_3H_7	CH_3	CH_3

tested clinically for the treatment of Parkinson's disease and other neuropsychiatric disorders[4–6]. Potent and selective activity of these compounds has been attributed to their agonist interactions with dopamine receptors in the mammalian central nervous system[2,7,8]. The clinical use of these compounds has been hampered by side effects (such as nausea), low oral absorption, chemical instability, and rather short duration of action. Effects to alleviate some of these problems has led to the development of several aporphine prodrugs in which the free catechol oxygens are bound to short-chain ether or ester groups. In particular, the compound methylenedioxy-N-*n*-propylnoraporphine (MDO-NPA, 3) provides for oral activity and somewhat extended duration of action, presumably via the slow metabolic conversion to the active free catechol, NPA, after prevention of its "first-pass" metabolism[6].

Numerous analytical approaches for the determination of either NPA or APO in biological samples include: gas chromatography (GC) with flame ionization detection (FID)[9–11], electron-capture detection (ECD)[12,13], or electron impact ionization mass spectrometry (MS)[14,15]; liquid chromatography (LC) with UV[16–20], fluorescent[21], or electrochemical detection (ED)[22–27]; and also thin-layer chromatography (TLC)[28–31], spectrophotometry[32], spectrofluorometry[33], radioenzymatic assay[34], and radioreceptor assay[6]. Each method possesses particular advantages and disadvantages, the choice of which depends largely on the required applications and detection limits and the instrumentation available. However, for the analysis of NPA at low concentration (ng/ml or ng/g) levels, which are pharmacologically significant, only the high-performance liquid chromatography (HPLC)–ED work of Sperk *et al.*[23] provides for linear detection at limits of less than 10 ppb*. For APO, detection limits below 10 ppb in biological matrices have been achieved using both HPLC–ED[22] and a non-linear radioreceptor assay[6].

The quantitation of drugs and metabolites at the trace level by GC–MS is an

* Throughout the article the American billion (10^9) is meant.

important analytical technique[35]. The studies of Watanabe *et al.* detailed the determination of APO in plasma[14] and brain[15] by conversion of APO to the bis(trimethylsilyl) derivative and subsequent analysis by packed column GC–electron impact ionization MS. Low detection limits for APO in plasma (30 ng/ml) and brain (50 ng/g) were achieved when NPA was used as an internal standard.

We now report a GC–MS method for the determination of NPA in rat serum and brain that employs fused-silica column gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICI-MS), an approach that provides the requisite sensitivity and selectivity for pharmacological studies. The precision and accuracy of quantitative GC–MS can be improved significantly through the use of stable isotope analogues as internal standards, for isotope dilution MS[36]. With the recent availability of multideuteriated aporphines of high isotopic purity[37], we have been able to develop an isotope dilution GC–MS assay of NPA in serum and brain tissue. Due to the trace levels of free NPA expected in animal tissues following treatment with typical mg/kg pharmacologic doses of NPA or its prodrug MDO–NPA, the demonstrated sensitivity of NICI-MS[38] was exploited. The conversion of NPA to the bis(trifuoroacetyl) derivative (4) was accomplished so as to provide an electrophoretic derivative necessary for this analysis.

EXPERIMENTAL

Reagents

Solvents used included methanol, toluene, and ethyl acetate of residue grade (J. T. Baker, Phillipsburg, NJ, U.S.A.). The following reagents were also obtained from Baker at the highest quality available: ascorbic acid, EDTA, phosphoric acid, sodium dihydrogenphosphate, disodium hydrogenphosphate, trisodium phosphate, and sodium hydroxide. The derivatization reagents trifluoroacetic anhydride (TFAA) and hexamethyldisilazane were obtained from Aldrich (Milwaukee, WI, U.S.A.). All of the aporphine compounds studied, including *R*-(−)-N-*n*-propylnorapomorphine hydrochloride (1a), *R*-(−)-10,11-methylenedioxy-N-*n*-propylnoraporphine hydrochloride (3a), *S*(+)-10,11-methylenedioxy-N-*n*-propylnoraporphine hydrochloride (3b), and $^2H_7$-*R*-(−)-N-*n*-propylnorapomorphine hydrobromide (5a), were synthesized and characterized in these laboratories. Rat serum and brain from untreated animals was obtained from Charles River Laboratories (Wilmington, MA, U.S.A.).

Glassware

Serum and brain extractions were conducted in glass culture tubes (100 × 15 mm) containing PTFE-lined screw caps. Brain homogenates were centrifuged in Corex (VWR Scientific, Boston, MA, U.S.A.) 15-ml centrifuge tubes. Derivatizations were carried out in conical glass Reacti-vials (Pierce, Rockville, IL, U.S.A.) (1.0 ml) equipped with screw caps containing PTFE septa. All glassware employed was initially cleaned by soaking in a detergent solution (RBS-35, Pierce) for 24 h, rinsed with deionized water and then methanol, and dried overnight at 110°C. The glassware was then subjected to a vapor phase silanization with hexamethyldisilazane[39] so as to minimize analyte losses due to surface adsorption.

Solutions

Stock solutions of NPA and [$^2H_7$]NPA were prepared, on a daily basis, by

weighing out 1.000–2.000 mg of material into a 0.5-dram glass vial. The solid was transferred to a 10-ml volumetric flask by adding to the vial aliquots of methanol (3 × 0.5 ml) and quantitatively transferring the methanol solution via glass pipette to the volumetric flask, and filling to the mark with an aqueous ascorbic acid solution (2 g/l). Working solutions of 1.00 ng/μl NPA and 1.00 ng/μl [$^2H_7$]NPA were prepared by making appropriate dilutions of the stock solutions using the ascorbic acid solution as diluent. All NPA solutions were kept chilled (5°C) and in the dark prior to use to minimize degradation.

Treatment of animals

Male Sprague–Dawley rats, 190–210 g body weight (Charles River, Wilmington, MA, U.S.A.), were used for the experiments. Rats were given various doses of *R*-(−)-MDO-NPA and *S*-(+)-MDO-NPA in isotonic saline solution (1:4, v/v) by intraperitoneal (i.p.) injection. The animals were killed by decapitation 30 min later and the tissues were rapidly removed, frozen on dry ice, and kept frozen at −70°C until analysis after initial separation of serum from clotted rich-wound blood by centrifugation at 1000 *g* at 4°C for 15 min.

Extraction of rat serum samples

An aliquot of thawed rat serum, 1.0 ml, was transferred to a silanized glass culture tube containing 100 μl of the ascorbic acid solution and 100 μl of a 10 g/l EDTA solution in 0.25 *M* sodium hydroxide. The tubes, kept chilled throughout sample preparation by immersion in an ice–water bath, were spiked with 50 μl of the [$^2H_7$]NPA internal standard solution. At this level of spiking, the internal standard [$^2H_7$]NPA was introduced at a concentration of 50 ng per ml of serum. Each tube was then vortexed for 30 s and allowed to equilibrate in the ice bath for 30 min.

Following equilibration, ethyl acetate (2.0 ml) and the phosphate buffer (2.0 ml) were added to each tube and the tubes were shaken on a rotary shaker for 5 min. The tubes were then centrifuged for 5 min to effect phase definition. The organic layer (upper) was transferred via a silanized glass pipette to a clean culture tube containing chilled 0.1 *M* hydrochloric acid (0.5 ml). The tubes were placed on a rotary shaker for 5 min, centrifuged, and the organic layer (upper) was discarded. To the aqueous layer was then added phosphate buffer (2.0 ml) and the pH of the resulting solution checked with indicator paper to be between 7.0 and 7.4. A pH adjustment, if required, was then made by dropwise addition of a 0.5 *N* trisodium phosphate solution. Next, ethyl acetate (1.0 ml) was added, the mixture placed on a rotary shaker for 5 min, and centrifuged. The organic layer (upper) then was transferred via silanized pipette to a silanized 1.0-ml Reacti-vial. All of the solvent was removed via a stream of nitrogen and to the dried residue was added the derivatization reagent TFAA (100 μl).

Derivatization was accomplished by placing the glass Reacti-vial in an aluminum block held at a temperature of 75°C for 60 min. Just prior to GC–MS analysis, the excess TFAA was removed via a nitrogen stream and toluene (25 μl) was added to dissolve the derivatized sample.

Extraction of rat brain samples

Individual rat brains, previously frozen, were transferred in dry ice, weighed on a balance to a precision of 0.01 g. The brain was then minced into ten or more pieces

and added to a chilled 15-ml Corex centrifuge tube containing 5.0 ml 0.1 *M* hydrochloric acid, 100 μl of the ascorbic acid solution, and 100 μl of the EDTA–sodium hydroxide solution. The tubes were spiked with 50 μl of the [$^2H_7$]NPA internal standard solution. At this level of spiking, the internal standard [$^2H_7$]NPA was introduced at an absolute amount of 50 ng or at a concentration of 25–35 ng per g of brain, depending on the net weight.

The contents of the centrifuge tubes were homogenized using a high-speed spinning PTFE-tipped rod for 60 s. The brain homogenates were then centrifuged at 12 000 *g* for 15 min. The clear supernatant from each tube was transferred to a clean culture tube and 3.0 ml ethyl acetate were added. The mixture was shaken for 10 min on the rotary shaker, centriguged for 10 min at 1200 *g*, and the upper organic layer was discarded. The pH of the aqueous solution was adjusted by dropwise addition of a 0.5 *M* trisodium phosphate solution to give a pH of 7.0–7.4, checked with pH paper. For a majority of samples this pH adjustment required 0.70 ml of base. Ethyl acetate (2.0 ml) was then added to the tube, which was then shaken for 10 min, and then centrifuged for 10 min at 1200 *g*.

The upper organic layer was again transferred to a 1.0-ml Reacti-vial, the ethyl acetate removed via a nitrogen stream, and 100 μl of TFAA were added to the residue. The derivatization was carried out at 75°C for 1 h. After allowing the vials to cool, the excess TFA was removed via a nitrogen stream, and 25 μl of toluene were added.

Gas chromatography–mass spectrometry

All GC–MS analyses were carried out on a Finnigan 4021 GC–MS system equipped with the pulsed positive ion negative ion chemical ionization (PPINICI) option. Control of the mass spectrometer and acquisition of data were carried out with the Finnigan-Incos data system and software. The Finnigan Model 9600 gas chromatograph was modified to accept a capillary on-column injector obtained from J&W Scientific (Rancho Cordova, CA, U.S.A.). A DB-5 fused-silica capillary column (15 m × 0.25 mm I.D.) (J&W Scientific) with a film thickness of 0.25 μm was employed for all analyses and was inserted directly into the ion source of the mass spectrometer. Helium (Matheson, UHP) was used as carrier gas, while methane (Matheson, UHP) serving as the CI reagent gas was admitted into the source to provide a pressure of 0.30 Torr. Mass spectrometer operating parameters included: 70 eV ionizing potential, 0.50 mA filament current, 250°C source temperature, and 1000 V electron multiplier voltage. Optimal mass spectrometer ion source tuning was conducted by admitting perfluorotri-*n*-butylamine (FC-43) into the source and monitoring the intensity and peak shape of an ion at *m/z* 395, a minor fragment ion of this calibrant compound. The GC oven initially was set at 95°C and , upon injection, ramped linearly to 250°C at a rate of 15°C/min. Under these conditions, the bis(trifluoroacetyl) derivative of NPA eluted at a temperature of 220°C with a retention time of 7.5 min. For the trace level analyses, selected ion monitoring data acquisition was accomplished by scanning on an equal basis for a total of 0.45 s over the two ions of interest, *m/z* 390 and *m/z* 397.

Calculation

Individual calibration curves were constructed for rat serum and brain by preparing drug-free serum and brain samples using the identical procedures as described for the treated samples, with the exception that known amounts of unlabeled

R-(−)-NPA were spiked into the samples prior to extraction. For serum, levels of 0, 1.0, 2.0, 5.0, 10, 20, 50, and 100 ng NPA per ml of serum were prepared. For brain, total fortifications of 0, 1.0, 2.0, 5.0, 10, 20, 50 and 100 ng NPA were added to individual brains (1.0–2.0 g wet weight).

Peak areas in the ion current profiles for NPA (*m/z* 390) and [$^2H_7$]NPA (*m/z* 397) were integrated using the Finnigan-Incos software. Calibration curves for both serum and brain were constructed by plotting the ion current ratios I_{390}/I_{397} *versus* the corresponding concentration ratios NPA/[$^2H_7$]NPA. Linear regression analysis on the resulting plots was performed to provide the constants for the equation $y = mx + b$ where m represents the slope and b the intercept of the curve. Concentrations of NPA in the treated samples were obtained by introducing the observed ion current ratio into the equation $C_{NPA} = (I_{390}/I_{397} - b)/m$ and solving for C_{NPA}.

As a check of the method's precision and accuracy, replicate blank rat serum samples were spiked at a level of 20 ng NPA per ml and replicate blank rat brain samples spiked at a total amount of 10 ng NPA per brain sample (1–2 g). Extraction and analysis were performed on these samples concurrently with samples from treated animals. To assess contributions of interferences from tissue matrix or reagents, blank samples were prepared and analyzed alongside samples from treated animals.

RESULTS AND DISCUSSION

The NICI mass spectrum of the bis(trifluoroacetyl) ester derivative of NPA (4) consists primarily of the fragment ions $[M-CF_3CO]^-$ at *m/z* 390 and $[CF_3CO]^-$ at *m/z* 113 (Fig. 1). The $[M-CF_3CO]^-$ ion from this electrophoretic derivative was

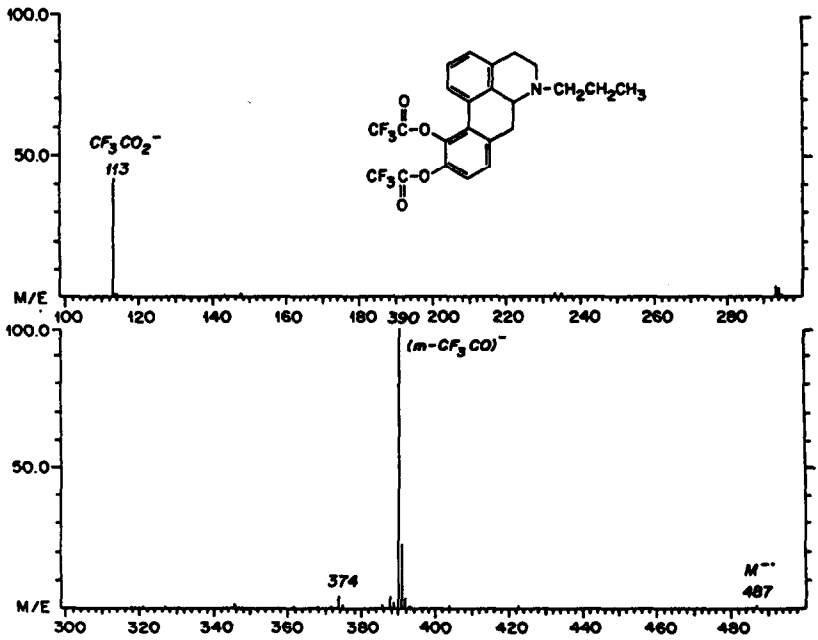


Fig. 1. Electron-capture negative ion CI mass spectrum of bis(TFA)-NPA.

found to provide both adequate sensitivity and specificity for the ceterminination of NPA at the required ppb levels in serum and brain. Picogram quantities of derivative 4 injected into the GC–MS system can be observed readily by operating under methane conditions with selected ion monitoring data acquisition. Stable isotope dilution with the internal standard [$^2H_7$]NPA (5) provided a reliable means of quantitation for NPA, as the NICI-MS spectrum of derivatized [$^2H_7$]NPA (6) gave the expected $[M-CF_3CO]^-$ ion at *m/z* 397.

The isolation and derivatization procedures for NPA were adapted largely from a GC–ECD method developed previously in these laboratories[12]. Extraction with ethyl acetate provided for high recoveries of NPA or APO from biological tissues when sample pH is within the optimum range of 6–8[10,17]. Furthermore, back-extraction into dilute hydrochloric acid has been employed to facilitate the removal of neutral and acidic components known to have detrimental effects on GC-based assays of NPA or APO in serum[9,12]. As degradation of the catechol NPA by oxidation to the corresponding quinone[40] has been a concern in these and related catecholamine[41] assays, precautions taken to reduce this occurrence included the use of ascorbic acid as an antioxidant and the use of EDTA[18,22].

Typical selected ion monitoring mass chromatograms are shown for a calibration sample of rat serum spiked at 1.0 ng/ml (Fig. 2), where the ion current traces at *m/z* 390 and *m/z* 397 are from the derivatives 4 and 6, respectively. With the fused-silica capillary column employed, the labeled analogue (6) eluted approximately 3 s before the unlabeled derivative (4) with a retention time of 7.5 min. Inspection of the ion current profiles obtained for all blank tissue samples revealed no interference at *m/z* 390 near the retention time of 4.

The linear regression analyses performed on the calibration curves generated using drug-free rat serum and brain extract revealed excellent linearity and are summarized in Table II. Method precision and accuracy were assessed by spiking

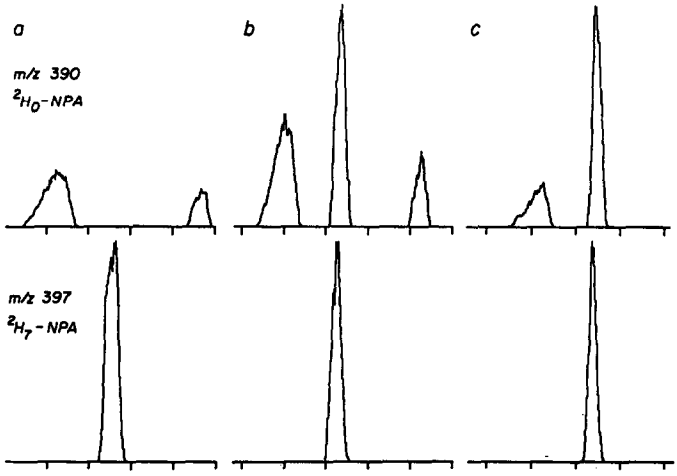


Fig. 2. Determination of NPA in rat serum. Mass chromatograms obtained from rat serum extracts at *m/z* 390 $[M-CF_3CO]^-$ for NPA and *m/z* 397 $[M-CF_3CO]^-$ for [$^2H_7$]NPA. (a) Blank serum sample. (b) Blank serum spiked at 20 ng/ml NPA. (c) Serum extract from a 3.0-mg/kg *R*-(−)-MDO-NPA treatment (4.2 ng/ml NPA observed in this sample). Note: 50 ng [$^2H_7$]NPA spiked in all serum samples; all mass chromatograms normalized to [$^2H_7$]NPA peaks.

TABLE II

LINEAR REGRESSION ANALYSIS OF NPA CALIBRATION CURVES

Matrix	*Linear regression constants**		
	m	*b*	r^2
Serum	1.30	−0.059	0.998
Brain	1.42	−0.056	0.996

* $y = mx + b$; $y = I_{390}/I_{397}$; x = NPA/[$^2H_7$]NPA.

replicate rat serum and brain samples with known levels of NPA and analyzing. The results (Table III) indicate a relative standard deviation of 4.2% at the 20 ng/ml level for rat serum ($n=5$) and 3.6% at the 10 ng level in rat brain ($n=3$). It should be noted that these spiking studies were not carried out alongside the calibration study, but were conducted on separate days with fresh solutions.

TABLE III

METHOD PRECISION AND ACCURACY

Matrix	*NPA spike*	*NPA calc.*	*S.D.*	*Rel. S.D.*	*n*
Serum	20 ng/ml	19.5 ng/ml	0.81	4.2%	5
Brain	10 ng/g	10.0 ng/g	0.36	3.6%	3

This method was utilized for pharmacological studies concerning the metabolic production of NPA *in vivo* following the administration of the prodrug MDO-NPA (6). Earlier work by Sperk *et al.*[23] and Maksoud *et al*[12] detected free catechol NPA in the tissue of animals treated with the prodrug MDO-NPA. The present study served to confirm these identifications, as the GC–MS data provide a more definite identification than either the HPLC–ED[23] or GC–ECD[12] methods.

TABLE IV

DETERMINATION OF NPA IN SERUM AND BRAIN AFTER TREATMENT WITH MDO-NPA

Treatment (mg/kg)	*NPA concentration**,**		*Brain/serum concentration ratio*
	Serum (ng/ml)	*Brain (ng/g)*	
R-(−)-MDO-NPA(1.0)	2.77 ± 0.10	19.6 ± 10.4	7.1
R-(−)-MDO-NPA(3.0)	7.64 ± 3.55	46.0 ± 25.1	6.0
R-(−)-MDO-NPA(10)	19.6 ± 2.30	114 ± 32.4	5.8
S-(+)-MDO-NPA(1.0)	14.1 ± 11.3	7.87 ± 4.59	0.56
S-(+)-MDO-NPA(3.0)	118 ± 15.3	38.1 ± 6.23	0.32
S-(+)-MDO-NPA(10)	118 ± 31.6	35.2 ± 8.70	0.30

* Animals sacrificed 30 min after i.p. injection of test agents.

** Concentrations represent the mean ± standard deviation from the separate analysis of three identically dosed animals.

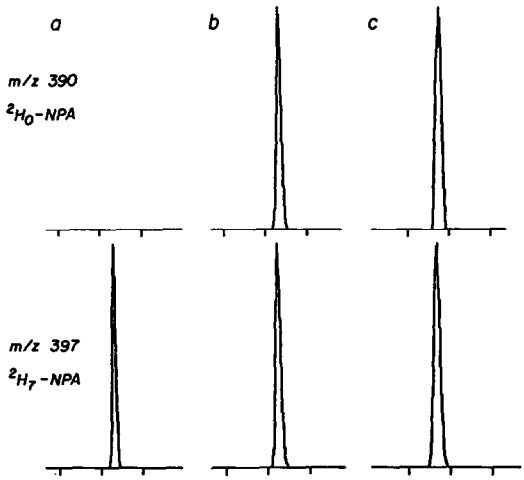


Fig. 3. Determination of NPA in rat brain. Mass chromatograms from rat brain extracts at *m/z* 390 $[M-CF_3CO]^-$ for NPA and *m/z* 397 $[M-CF_3CO]^-$ for $[^2H_7]$NPA. (a) Blank brain sample. (b) Blank brain spiked at 14 ng/g NPA. (c) Brain extract from a 3.0-mg/kg *R*-(−)-MDO-NPA treatment (21 ng/g NPA observed in this sample). Note: 50 ng $[^2H_7]$NPA spiked in all brain samples (1–2 g), and mass chromatograms normalized to $[^2H_7]$NPA peaks.

Another biological question we wished to explore in utilizing the GC–NICI-MS method was a comparison of resultant NPA levels in the rat after treatment with either stereoisomer *R*-(−)-MDO–NPA (3a) or *S*-(+)-MDO–NPA (3b). Previous pharmacological studies have shown marked differences in the behavior of rats treated with *R*-(−)-NPA (1a) *versus* *S*-(+)-NPA (1b), their MDO prodrugs, or with *R*-(−)-APO (2a) *versus* *S*-(+)-APO (2b)[42]. In this study, the stereoisomers *R*-(−)-MDO–NPA and *S*-(+)-MDO–NPA were administered to rats at three dose levels, 1.0, 3.0, and 10 mg/kg (i.p.). The animals were sacrificed 30 min later and the serum and brain analyzed for the presence of free NPA, the presumed bioactive form of MDO-NPA. In Table IV are presented the results of this experiment, and representative selected ion monitoring mass chromatograms for both serum and brain extracts from animals treated in this study are shown in Figs. 2 and 3, respectively.

Distinct differences in the tissue concentrations and distribution between serum and brain tissue of free *R*-(−)-NPA and *S*-(+)-NPA were observed. At all three dose levels, a concentration of *R*-(−)-NPA predominated in the brain (brain/serum) ratios between 5.8 and 7.1) while concentration of *S*-(+)-NPA favored serum (brain/serum ratios between 0.30 and 0.56). These differences may arise from isomeric selectivity in the distribution of NPA between serum and brain or to isomeric differences in the metabolism of MDO-NPA by enzymatic O-dealkylation conversion of MDO-NPA to free NPA. An analogous situation was observed for the regiospecific and stereoselective microbial O-dealkylation of 10,11-dimethoxy-NPA (7) reported by Neumeyer *et al.*[43]. Work is currently in progress to clarify this phenomenon.

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