Using HPLC-ECD for the Comparison between Effects of Tranylcypromine and LY134046 on the Brain Adrenaline and Noradrenaline

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ABSTRACT: The effects and turnover of central adrenaline (Ad) has not been studied comprehensively as of noradrenaline (NA) and other amines. Effects both of the monoamine oxidase (MAO)- and phenylethanolamine N- methyl transferase (PNMT)- inhibitors were evaluated on the levels of Ad of certain brain regions, and compared with that of NA. The levels of the amines and metabolite in brain tissue extracts of male Wister rats i.p. administered either LY134046 or tranylcypromine were measured by high performance liquid chromatography- electrochemical detector (HPLC-ECD) and their changes from the controls were examined using the Student's unpaired t-test. The detection of Ad was at 2 nanoamper (nA) sensitivity, while of NA 3,4-dihydroxyphenyl acetic acid (DOPAC) and dopamine was at 10nA (LY134046) or 20nA (tranylcypromine). LY134046 causes significant decrease in the Ad levels of hypothalamus (-47%) and of brainstem (-73%) but was not detected in hippocampus or striatum both of the LY134046 and the controls. In contrast LY134046 causes significant increase in the dopamine and DOPAC of all tested brain regions, and in the NA of hypothalamus only (+70%). Tranylcypromine significantly increases the levels of NA and dopamine in all tested brain regions, whereas the Ad levels only increased in hypothalamus (+58%) and brainstem (+67%) but it was below the detection level in hippocampus or striatum. The results indicates much higher tissue levels of the NA and DOPAC substances compared with the Ad and confirm the suggestion that the pharmacology of central Ad is analogous to that of central NA, with the exception of its synthesis. The significant decrease in the DOPAC of all brain regions though is more difficult to explain it could indicate that the LY134046 might also act on dopamine uptake sites which needs to be investigated.

Keywords: LY134046, tranylcypromine, Ad, NA, dopamine, hypothalamus, brainstem.

استخدام HPLC-ECD لمقارنة تأثير التراثيل سايبرومين وLY134046 على كل من ادرئنالين ونورادرنين الدماغ

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Introduction

There are two responsible enzymes in catabolism of catecholamines (CAs): monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT). MAO an iron- and a flavine- containing enzyme is located in the outer mitochondrial membrane and exhibited in virtually all tissues of mammals catalyzing the oxidative deamination of monoamines (1,2).

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There are two isoforms of MAO, MAO-A and MAO-B which have different affinities for various substrates including both important neurotransmitters, hormones, and susceptibility to inhibitors.

MAO-A preferentially deaminates serotonin, melatonin, adrenaline (Ad), and noradrenaline (NA).

MAO-B preferentially deaminates phenylethylamine and trace amines like biologically active dietary amines such as tyramine.

Dopamine is equally deaminated by both types (1,2) the major metabolite produced from degradation of NA and Ad following neuronal re-uptake are 3,4-dihydroxyphenylethylglycol (DHPG) and 3-methoxy-4-hydroxyphenylethylglycol (MHPG) or 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in case of dopamine ( 3 , 4 ). MAO-inhibitors (MAOIs) are chemicals which inhibit the activity of the MAO enzyme family thus, preventing the breakdown of monoamines and thereby increasing their availability. Tranylcypromine is a non-hydrazine MAOI. It is structurally
related to amphetamine to which it is metabolized in overdose (5).

In the brain metabolism of Ad is predominantly by MOA type A (6,7). The well-established involvement of NA of the hypothalamic adrenergic terminals of the A1- and A2- brainstem cell groups in the release of vasopressin (AVP) provide support for Ad involvement in this function, since at least in rat forebrain Ad appears to be found primarily co-stored in noradrenergic nerve endings (8). Moreover, the regulation and the metabolism of Ad and NA is very similar as they both regulated by the same receptors and end by formation of the same metabolites (6,7). The pharmacology of the central Ad however, has not been studied as comprehensively as that of NA, primarily for two reasons: First the relatively low concentration of Ad (compared with NA) in hypothalamic adrenergic nerve ending, second specificity of most drugs which affect NA release or metabolism also alter Ad (8). Though, the most selective method of altering Ad content is to select inhibit its synthesis. In his comprehensive review of the pharmacological manipulation of brain Ad, Fuller (9) stated that only inhibition of the enzyme phenylethanolamine N-methyltransferase (PNMT) would decrease Ad formation without decreasing NA or dopamine formation, since in the synthesis pathway of Ad in vivo from the amino acid L-tyrosine, it is the enzyme which is catalyzing the final step of Ad biosynthesis from NA (9).

In view of the finding of a comparable decrease in the Ad and AVP levels in rat regions, brainstem, and hypothalamus following to i.p. administration of PNMT-inhibitor LY134046 and all the PNMT-inhibitors share structural features with MAO-inhibitors and in having a variety of behavioral effects in the rat (6,8,10,11,12) the intention of this study was to evaluate the effects of the intraperitoneally (i.p.) administered by both non-selective MAO-inhibitor, tranylcypromine and the PNMT-inhibitor 8, 9-dichloro-2, 3, 4,5-tetrahydro-1H-2-benzazepine, (LY134046) on the levels of Ad of the hypothalamus, brain stem, hippocampus, and striatum comparing it with the effects of NA and other amines using high performance liquid chromatography-electrochemical detector (HPLC-ECD) for these measurements.

Materials and Methods

The experiments were performed on male Wister rats weighting approximately 150-210 g maintained under under 12h light : 12h dark photoperiod with food and water available and libitum. The rats were divided into three groups each of 6 administered i.p. either with 40mg/kg B.W. LY134046 (EliLilly), 10mg/kg B.W. tranylcypromine (Smith, Kline & Frenchs), or normal saline (0.9% w/v). Rats were decapitated 4 hours later and the brain was quickly removed and placed on a cool tray after the removal of the neurointermediate lobe of pituitary gland (NIL) and median emenance (ME), coronal brain slices were cut by hand to dissect out hypothalamus, brainstem, hippocampus and striatum using the method of Palkovits and Brownstein (13).

The NIL was separated from the pituitary’s two anterior lobes, while the gland was still in place at the base of the skull by removing the thin membrane that covered and fixed it to the skull, and
picked up using small watch markers forceps. The ME was dissected from the top of the hypothalamus with fine, sharp, and curved forceps by placing the two curved shanks of the forceps on either side of the ME (into the infundibular sulcus) and with a gentle press against and close to the base of the hypothalamus the ME was plucked out. The whole hypothalamus was dissected as the region beginning approximately 0.6-0.7mm behind the bregma and ending at the level of the mamillary body in the midbrain at P4500µm level (Figure 1). One cut was made rostral to the optic chiasma at zero (bregma) level and the second just to the end of the hypothalamus as far ventral as the end of the third ventricle at P4500µm level. The brain portion situated between P10800-14700µm was dissected as brainstem region containing several 'cardiovascular sites' rich in Ad and NA forming neurons including nucleus tractus solitarius (NTS) and ventral lateral medulla (VLM) which contain the rostral VLM/C1 Ad-neurons and caudal VLM/A1 NA-neurons (Figure 1).

A primary coronal cut was made between P1800µm and P6600µm to dissect out the hippocampal complex region by opening and removing the fold which lies under the cerebral cortex (Figure 2). Striatum consists of two areas the ventral pocket and the nucleus accumbens. A primary coronal cut was made between A300-0µm to dissect out and use only the ventral pocket of striatum (Figure 2). The dissected tissue from rats was treated with LY134046 and those were treated with tranylcypromine was divided into approximately equal halves. One half was immediately extracted for AVP and the dried extract was stored at -80°C until measurement, while the other half was stored at -80°C for subsequent measurement of CA by HPLC-ECD. Extraction of CAs from brain samples following that was carried out by Adams and Marsden (14). Stored brain samples were weighed and sonicated for 10-15sec (Ultra-sonics Soniprep-150) then centrifuged for 5min (3000g, at 0-4°C: Mistral 6000, Fisons, England). The supernatant was removed and added to an eppindorf tube containing activated Woelm alumina together with ice cold 1.5M Tris HCl, containing 0.1 mM diaminooxothane-tetra-acetic acid disodium salt (EDTA) which was adjusted to pH 8.6. The eppendorfs were capped and shaken for 5min to allow the CAs to be absorbed onto the alumina. The samples were spun in microcentrifuge and then the supernatant was removed and discarded. Cold double deionized distilled water (DDD) was added and the tube was shaken before spinning in microcentrifuge for 1min. All the supernatant was carefully removed and ice cold 0.1M perchloric acid (PCA) containing 0.4mM sodium metabisulphite (NaMS) was added and the tubes were shaken for 2min before spinning in the microcentrifuge to re-extracted the CAs from the alumina to give a final brain tissue samples ready for injection onto the HPLC column. The obtained pellet after the initial centrifugation was stored at -20°C until the protein content was determined by Lowry et al. method (15).

The used HPLC-ECD contained Altex 110A Solvent Metering System with an analytical liquid head delivers from 5000-6000 psi. Reodyne modle 7125 syringe manual sample injector and loop approximately of 500µl was used. The used reagents of mobile phase; sodium hypo-sulphate (NaH2PO4), sodium octyl-sulphate (ion paring reagent),
EDTA, and methanol, were all of HPLC grades (Fisons). Ultra pure DDD water were used in the preparation of the mobile phase which adjusted to pH4.1 before it reached the final volume it is filtered and degassed in a sonic water bath. The mobile phase was recycled and allowed to equilibrate for at least 1hr before injection of a standards containing NA, Ad, dopamine, DOPAC, 5-hydroxy-indol-3-acetic acid (5HIAA) and HVA (all at $10^{-7}$ M). Reversed phase ion pair (RP-IP) chromatography was used. Analyses were partitioned between a hydrophobic stationary phase and aqueous mobile phase. Solutes were eluted in order to decrease in polarity (increasing hydrophobicity) in 4.6mm×15cm long ultrasphere column (Beckman) packed with C18 octadecylsilyl (ODS-5µm) groups bounded to the silica surface. The CAs and their metabolites were detected by their oxidation at a carbon-based electrode following the separation using LC-4A Amperometric Bioanalytical System Detector with a single glassy carbon working electrode. The reference electrode was an Ag/AgCl electrode with 3M sodium chloride. The resultant current of oxidation being directly proportional to the number of molecules oxidized, and the output was amplified and displayed on the servoscribe potentiometric recorder as a trace. The peak areas and elutes concentration were calculated manually. $2\times10^{-7}$ M 3,4-dihydroxy-benzylamine was added to the standard stock solution as well as to the brain tissue at the time of extraction to calculate the percentage recovery of the CAs from brain tissue samples after extraction. The determined retention time of series of the standard CAs and metabolite (calibration curve for each constricted between 1 and 100 pmoles injected onto the column) were compared with that of the brain tissue samples, and calculated in pmoles/g tissue.

Adrenaline ((+)-bitartrate salt), noradrenaline ((-)-bitartrate salt), dopamine (3-hydroxytyramine), DOPAC, 5HIAA, HVA, and 3,4-dihydroxybenzylamine all from Sigma.

**Data Analysis**

The levels of the amines and metabolite in brain tissue extracts in all experiments are expressed as pmoles/g tissue. Differences between amine and metabolite levels in the presence of the drug and the control were examined using the student's unpaired t-test. Dorsal and saggital views of the surface of the rat brain are showing the slices taken in the present study. The location of each slice 1-3 was taken according to external regional landmarks and as described by Palkovits and Brownstein (1988). The regions dissected out from each slice are shown by the dotted lines nso, supraoptic nucleus; npy, paraventricular nucleus; CO, optic chiasma; TO, optic nerve; nts, nucleus tractus solitaries; pgi, paragigantocellular reticular nucleus of the ventral lateral medulla.
Figure 1: Dissected hypothalamus beginning 0.6-0.7mm behind the bregma

Figure 2: The hippocampal was dissected out by opening and removing the fold

Dorsal and sagittal view of the surface of the rat brain showing the slices taken in the present study. The location slices 1-2 was taken according to external regional landmarks and as described by Palkovits and Brownstein (1988). The regions dissected out from each slice are shown by the dotted lines: s, subiculum; GD, dentate gyrus; HF, hippocampal fissure; HI1, hippocampus CA1; HI2, hippocampus CA2; HI3, hippocampus CA3; HI4,
Results and Discussion

Initial studies were concerned with obtaining clear chromatographic separation between the constituents of the mixed standards. The desired separation was achieved by doubling the amount of ion pairing agent from 110mg to 220mg/l. Modification of the pH for reverse phase chromatography altered the retention: an increase in pH caused a decrease in the retention of the acid metabolites but had no effect on the retention times of the amines or their non-acid metabolites and vice versa. In addition, the effect of the % methanol of the mobile phase on the retention times of the selected standards was also measured to determine the optimum conditions for separation of the CAs and their metabolites. The final mobile phase formula provided sharp and distinctive peaks for the standards contained 0.15M NaH2PO4, 0.2mM sodium octyl-sulphate, 0.1mM EDTA and 9% methanol of pH4.1.

The results show that isocratic chromatographic separation adequately separated all the amine and metabolite standards tested with reference to their retention times (Figure 3). Calibration graphs were constructed for 5 doses of the amine and metabolite standards; NA, Ad, DOPAC, dopamine, 5HIAA, and HVA (10^-6-10^-8M) and the samples were assayed by HPLC-D-ECD as described previously. Characterization of peaks observed in brain tissue extracts were made by comparing their HPLC retention time with the retention time of amine and metabolite standards. Figures (4,5,6,7) are examples of a typical HPLC trace demonstrating the separation of the amines and the acid metabolite in rat hypothalamus and brainstem extracted from animals treated with LY134046 (Figures 4,5) and tranylcypromine (Figures 6,7). The figures show that Ad was detected using 2 nanoampair (nA) sensitivity setting to give peaks >3:1 signal to noise, while NA, DOPAC, and dopamine were measured using sensitivity of 10nA from tissue of animals treated with LY134046 or 20nA from tissue of animals treated with tranylcypromine, indicating the much higher tissue levels of the latter substances. Table1 shows the change in Ad, NA, dopamine and DOPAC levels in rat brain regions; hypothalamus, brainstem, hippocampus, and striatum 4hr after administration of tranylcypromine (10mg/kg,i.p.). Ad levels in hypothalamus (+58%) and brainstem (+67%) were significantly elevated, while Ad was below the level of detection in the hippocampus or striatum in both control and treated animals. NA and dopamine were also significantly increased in the whole brain regions, whereas DOPAC was significantly decreased. In table 1 also are results obtained for Ad, NA, dopamine, and DOPAC concentration in regions of the rat brain: hypothalamus, brainstem, hippocampus, and striatum 4 hrs following injection of 40mg/kg LY134046 i.p. The Ad level was significantly decreased in the hypothalamus (-47%) and brainstem (-73%), but was below the level of detection in hippocampus and striatum in both control and LY134046 treated animals. In a similar manner to Ad, DOPAC was significantly decreased in all the areas studied. In contrast, the tissue level of NA was
significantly increased (+70%) by LY134046 in the hypothalamus but not in the other regions, whereas the tissue level of dopamine was significantly increased in all the regions except the hippocampus.

The aim of this study was to compare the presence and turnover of the central Ad with that of NA, dopamine, and DOPAC by measuring the change in their levels in brain levels following administration of tranylcypromine or LY134046. The effects of tranylcypromine are in close agreement with previous studies (7,12,16,17), which demonstrated tranylcypromine increases tissue amine levels probably by acting as a suicide substrate for MAO. Such action \textit{in vivo} is dependent upon it being metabolized to form a reaction intermediate which then covalently attaches to the MAO molecule preventing amine degradation (18). Previous studies have shown that oxidative deamination is an important metabolic pathway by which Ad is degraded and type A MAO enzyme is responsible for this oxidation (16,19). MAO however exists at least into two forms type A&B which differ in their substrate specificity and susceptibility to inhibitors (1). Evidence from clinical trials on the effectiveness of MAO-inhibitors such as tranylcypromine, in treatment of depression, has lead to the proposal that the selective inhibition of MAO type A may have more antidepressant efficacy than inhibition of MAO-B (7). A very recent review by Lum and Stahl (12), highlighted presence of at least 11 brain regions associated with depression, involved neurons in circuits with different functions, and thus different symptoms regulated by information processing in unique brain areas regulated in turn by monoamine input for 5-HT, NE, and DA coming from the brainstem. All of these three neurotransmitters can be broken down by the enzyme MAO-A which is located on the mitochondria of the presynaptic neuron, as well as other neurons and glia. The enzyme MAO-A that degrades the neurotransmitters, may be overactive in patients with depression (12). Additional research has traced the high MAO-A activity to a protein that controls the genetic expression of MAO-A, known as R1 which is a repressor protein represses the genetic machinery that leads to the production of MAO-A (20).

The increase in the levels of Ad in many of the 11-depression associated brain regions that have been studied in this investigation, highlighted a possible role for central Ad in depression further to the above mentioned three neurotransmitters, since Ad is selective substrate for MAO-A (17), and at least in rat forebrain, Ad appears to be found either stored in adrenergic fibers projected from adrenaline forming neurons in brainstem, or primarily co-stored in noradrenergic nerve endings (8) which together suggests possible involvement of Ad in this effect. This is of particular interest since a low level of cerebral spinal fluid Ad have been reported in patients with anti-depression, which increased approximately 4-fold (while NA concentrations were unchanged) following therapy with MAO-inhibitors (21). A possible role of CAs in the regulation of BP has also been reported. One is the antihypertensive action of MAO-inhibitors in humans and animals (22,23) which may resulted from NA accumulating at inhibitory \(\alpha\)-adrenoceptors in brain. These studies however do not differentiate between
effects on NA or Ad and the possibility remains that the elevation of brain Ad following administration of these MAO inhibitors to human patients plays a part in the antihypertensive effects.

The significant decrease of Ad after LY134046 administration in another trail of this study, is also consistent with the finding of other studies demonstrating marked depletion of central Ad after PNMT inhibition (6,9,24). The PNMT inhibitors are the only known drugs selectively to decrease Ad synthesis, without altering the synthesis of other amines (22,24). The decrease in Ad was not accompanied by a marked increase in NA (except in hypothalamus), in contrast to dopamine which increased significantly in all studied regions. The alteration in dopamine is in agreement with finding of transient increase in dopamine (but not NA) following LY134046 administration (6). As LY134046 is also α2-adrenoceptor antagonist (25) it increases the production and activity of tyrosine hydroxylase and dopamine β-hydroxylase the enzymes responsible for synthesis of dopamine and NA respectively (26). An explanation for the increase in NA and dopamine in the present study therefore, is that the α2-adrenoceptor blocking activity of LY134046 increased release and synthesis of NA from nerve terminals via removal of the auto-receptor mediate feed-back control (27). The significant decrease in DOPAC in all brain regions is more difficult to explain but could indicate that the LY134046 might also act on dopamine uptake sites, but this needs to be investigated. In conclusion our results are the first in vivo data demonstrating a significant increase in central NA and dopamine with a concomitant decrease in DOPAC following administration of the PNMT-inhibitor, besides it confirms the suggestion that the pharmacology of central Ad, is analogous to that of central NA with the exception of its synthesis.Effects of the LY134046 (40mg/kg i.p.) and tranylcypromine (10mg/kg i.p.) on adrenaline, noradrenaline, dopamine and DOPAC levels in rat brain regions; hypothalamus,brainstem, hippocampus, and striatum. Results (pmoles/g tissue) given as mean ± SEM of 6 rats/group. *p<0.05, **p<0.01 and ***p<0.001 significant differences from control group by Student's t-test. ND= not detected level at HPLC-ECD conditions described in table (1).
Table 1: show significant differences from control group by students t-test

<table>
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<th>Brain regions</th>
<th>Adrenaline (Pmoles/g tissue)</th>
<th>Noradrenaline (Pmoles/g tissue)</th>
<th>Dopamine (Pmoles/g tissue)</th>
<th>DOPAC (Pmoles/g tissue)</th>
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<tr>
<td>Saline</td>
<td>289±44</td>
<td>6450±657</td>
<td>977±90</td>
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<td>158±31*</td>
<td>10980±1348*</td>
<td>2006±209***</td>
<td>458±64**</td>
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<tr>
<td>(-47%)</td>
<td>(+70%)</td>
<td>(+10%)</td>
<td>(-54%)</td>
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<td>7791±557</td>
<td>1164±90</td>
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<td>367.2±48*</td>
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<td>(+45%)</td>
<td>(-50%)</td>
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Figure 3: The isocratic chromatographic separation all the amine and metabolic standards

A typical trace of chromatographic separation and measurement by HPLC-ECD, of a 10 pmoles (injected on the column) standard of (1) NA, (2) Ad, (3) DOPAC, (4) DHBA, (5) dopamine, (6) 5HIAA or (7) HVA showing their retention times 6.5, 10, 12, 15.5, 25, 27 and 35 min respectively.

Figure 4: Example of HPLC-ECD typical traces obtained from a regional rat brain, hypothalamus

Example of HPLC-ECD typical traces obtained from a regional rat brain, hypothalamus i.p. treated either with saline (0.2 ml/ 100g) or PNMT-inhibitor, LY134046 (40mg/kg). Note that adrenaline can only be detected at 2nA and below, while the best detection of noradrenaline and dopamine was at 10nA.
Example of HPLC-ECD typical traces obtained from a regional rat brain and brainstem i.p. treated either with saline (0.2ml/100g), or PNMT inhibitor LY134046 (40mg/kg). Note that adrenaline can only be detected at 2nA and below, while the best detection of noradrenaline and dopamine was at 10nA.

Figure 6: Example of HPLC-ECD typical traces obtained from a regional rat brain and hypothalamus
Example of HPLC-ECD typical traces obtained from a regional rat brain and hypothalamus i.p. treated either with saline (0.2ml/100g) or MAO-inhibitor tranylcypromine (10mg/kg). Note that adrenaline can only be detected at 2nA and below, while the best detection of noradrenaline and dopamine was at 10nA or 20nA.

Example of HPLC-ECD typical traces obtained from rat brain region and brainstem i.p. treated with saline (0.2ml/100g), or MAO inhibitor, tranylcypromine (10mg/kg). Note that adrenaline can only detected at 2nA and below, while the best detection of noradrenaline and dopamine was at 10nA and 10nA or 20nA respectively.

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