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ORIGINAL ARTICLE

Differential metabolism of alprazolam by liver and brain cytochrome (P4503A) to pharmacologically active metabolite

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ABSTRACT

Cytochrome P450 (P450) is a superfamily of enzymes which mediates metabolism of xenobiotics including drugs. Alprazolam, an anti-anxiety agent, is metabolized in rat and human liver by P4503A1 and P4503A4 respectively, to 4-hydroxy alprazolam (4-OHALP, pharmacologically less active) and α-hydroxy alprazolam (α-OHALP, pharmacologically more active). We examined P450 mediated metabolism of alprazolam by rat and human brain microsomes and observed that the relative amount of α-OHALP formed in brain was higher than liver. This biotransformation was mediated by a P450 isoform belonging to P4503A subfamily, which is constitutively expressed in neuronal cells in rat and human brain. The formation of larger amounts of α-OHALP in neurons points to local modulation of pharmacological activity in brain, at the site of action of the anti-anxiety drug. Since hydroxy metabolites of alprazolam are hydrophilic and not easily cleared through blood-CSF barrier, α-OHALP would potentially have a longer half-life in brain.
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INTRODUCTION

Cytochromes P450 (E.C. 1.14.14.1; P450) and associated mono-oxygenases are one of the most important class of drug metabolizing enzymes in the body.[1] This family of heme proteins exists in multiple forms having distinct yet overlapping, substrate specificities and are encoded by a supergene family, several members of which have been well characterized.[2] The multiple forms of P450 are distinct gene products and have been classified into 36 gene families. The individual P450s are represented by an Arabic number denoting the family, followed by an alphabetical letter designating the sub-family and an Arabic numeral representing the individual gene within the subfamily.[2] Thus, the individual hepatic isoforms P4503A4 present in human and P4503A1 in rat both belong to the P4503A sub-family. P450 dependent mono-oxygenases are found predominantly in the microsomal fraction of tissues, but such activities are also present in mitochondrial fraction. The microsomal P450 mono-oxygenase enzyme system (which is of present interest) has three major components: (a) the heme protein, cytochrome P450 (P450); (b) the flavoprotein, NADPH cytochrome P450 reductase (reductase); and (c) phospholipid. Reductase helps the transfer of electrons from NADPH to the heme protein and is an essential component of the P450 monooxygenase system.

P450 mediated metabolism can lead to the detoxification of drugs rendering them pharmacologically inactive, or on the other hand, bioactivation can also take place wherein a drug can be metabolized to pharmacologically active metabolite[3] which may have longer or shorter half life compared to parent drug. Metab-

olism may be necessary for pharmacological action, if metabolite is active, and if metabolite is inactive, this would result in cessation of drug action.

Psychoactive drugs, such as antidepressants and neuroleptics are metabolized by hepatic P450. Specific forms of hepatic P450s such as those belonging to 3A, 2D, 2C and 1A subfamilies are involved in the metabolism of some of these drugs in both rat and human liver.[4,5] The major differences that are seen between the rat and human hepatic P450s is the distribution of the multiple forms. For example, amongst the isoforms of P450 belonging to the P4503A subfamily, P4503A4 is the predominant form of P450 in human liver and can account for nearly 60% of the total P450 present. In the rat liver, the analogous P4503A1 represents a relatively minor form of P450.[6]

It has long been appreciated that the liver is quantitatively the major organ involved in metabolic disposition of most xenobiotics, *in vivo*.[7] However, the importance of extrahepatic metabolism has been increasingly recognized especially during the past 2 decades. This has prompted extensive investigations into the xenobiotic metabolizing capability of extrahepatic organs in laboratory animals such as rat. Kidney, lung and nasal epithelium that are anatomically disposed to high levels of exposure to potential toxicants have been studied in rats.[8] The preferential localization of drug metabolizing enzymes within specific cell types in these organs renders such cells significant capability to metabolize drugs.[9] Thus, even minor metabolic pathways of xenobiotic metabolism can produce significant effects if it takes place at the site of action. These observations have prompted investigation into P450 associated mono-oxygenases in brain, with an effort to determine the capability of the brain to metabolize psychoactive drugs.[10]

P450 mediated metabolism of psychoactive drugs directly in the brain may lead to local pharmacological modulation at the site of action and result in variable drug response. Plasma drug levels which are often used as indicators of bioavailability of a drug reflect the variability in hepatic drug metabolism. However, it is being increasingly recognized that: (i) significant number of patients with adequate plasma levels do not respond to psychoactive drugs; and (ii) patients with acceptable plasma levels of drugs exhibit toxic side effects due to the drug. Thus, plasma levels of drugs are not always effective indicators of therapeutic outcome.[11]

Recent studies from our laboratory and others have demonstrated the presence of a competent microsomal P450 system in the rodent[12] and human[13] brain and its ability to metabolize a variety of xenobiotics. The appearance of multiple forms of P450 in brain and their selective inducibility by phenobarbital, 3-methylcholanthrene, β-naphthoflavone and ethanol have also been identified.[10] However, we know little about the ability of the brain P450 to metabolize psychoactive drugs and the potential consequence of such biotransformation at the site of action of these drugs.

Alprazolam is an extensively used anti-anxiety drug. In rat and human liver, it is biotransformed to hydroxylated metabolites, 4-hydroxy alprazolam (4-OHALP) and α-hydroxy alprazolam (α-OHALP). While, α-OHALP, the minor metabolite is pharmacologically more active, 4-OHALP, the major metabolite is less active.[14] This offered us an unique opportunity to test whether pharmacologically active metabolite(s) were formed in brain and more importantly if the metabolic profile differed between brain and liver, since hepatic biotransformation pathways cannot be often extrapolated to the brain. We, therefore examined the metabolism of alprazolam by rat liver and brain microsomes and compared it with the activity in microsomes prepared from human brain regions obtained at autopsy.

RESULTS

Metabolism of Alprazolam by Rat Brain Microsomes

Rat brain microsomes metabolized alprazolam to 4-hydroxy and α-hydroxy alprazolam. The activities were 5.44 $\pm$ 0.134 pmoles of 4-hydroxy alprazolam formed min^{-1} mg^{-1} protein and 2.42 $\pm$ 0.16 pmoles of α-hydroxy alprazolam formed min^{-1} mg^{-1} protein ($n = 5$ sets of microsomes prepared from pooled brains of ten rats). There was no detectable hydroxylation of alprazolam in incubations that did not contain NADPH or substrate. The 4-hydroxylase activity in control rat brain microsomes increased linearly over a protein concentration of 0.5–2 mg per ml incubation and over a time period of 5–30 min, but declined subsequently due to the instability of the metabolite. The metabolism of alprazolam to α-hydroxy alprazolam was linear over a protein concentration of 0.5–2 mg of microsomal protein ml^{-1} incubation however, the amount of metabolite formed decreased by over 50% when the incubation time was increased from 15–30 min due to the known instability of the metabolite. The amount of metabolite formed was constant from 5–15 min (data not shown). All incubations were therefore performed for 15 min and the metabolite(s) were assayed by HPLC within 30 min after completion of incubation. The Km and Vmax for the formation of 4-hydroxy and α-hydroxy alprazolam are given in Table 1. The rate of formation of 4-hydroxy alprazolam was about three-fold higher than that for α-hydroxy metabolite.

Distribution of Alprazolam Hydroxylase Activity in Human Brain Regions

We examined hydroxylation of alprazolam by different regions of human brain (cortex, cerebellum, straitum and

Table 1 Apparent Km and Vmax for the hydroxylation of alprazolam to its hydroxylated metabolites by rat and human brain microsomes

	α-OH ALP		*4-OH ALP*	
	K_m	V_{max}	K_m	V_{max}
Rat	0.211	2.03	0.56	7.48
Subject I	8.868	3.37	0.83	9.07
Subject II	3.56	12.77	3.2	21.96
Subject III	5.79	16.52	3.39	26.29

Apparent K_m and V_{max} calculated from Eadie–Hofstee plot are expressed as mM and picomoles of α-hydroxy alprazolam (α-OHALP) and 4-hydroxy alprazolam (4-OHALP) formed $min^{-1}mg^{-1}$ protein, respectively.

thalamus) as depicted in Figure 1. Maximal activity was seen in the striatum for both the metabolites. All the brain regions examined were capable of metabolizing alprazolam to both the hydroxylated metabolites. The activity varied from 2.4–4.9 pmoles of 4-hydroxy alprazolam formed min^{-1} mg^{-1} protein and 0.7–0.96 pmoles of α-hydroxy alprazolam formed min^{-1} mg^{-1} protein. No significant differences were seen amongst the brain regions examined. The human brain microsomal P450 mediated formation of 4-hydroxy alprazolam was linear through a protein concentration of 0.5–2 mg protein ml^{-1} and over a time period of 5–30 min. The formation of α-hydroxy alprazolam increased linearly when the protein concentration was increased from 0.5–2 mg. However, the activity was linear only over a short time period of 5–15 min and thereafter remained nearly constant up to 30 min. The Km and Vmax for the formation of both the hydroxylated metabolites by human brain cortical microsomes is given in Table 1. Significant inter–individual differences were noted in the apparent affinity and velocity constants for the formation of the hydroxylated metabolites amongst the different human brains examined. A common feature was the relatively low affinity (Km = 0.8–8.8 mM) for the alprazolam. However, this needs to be interpreted with caution, since the brain microsomes used for the assay of mono-oxygenase activity have contributions from both the glial and neuronal cells (unlike in liver) although the P450 is selectively localized in specific cell population. Studies with the purified enzyme would yield more accurate information on the kinetics of the enzyme.

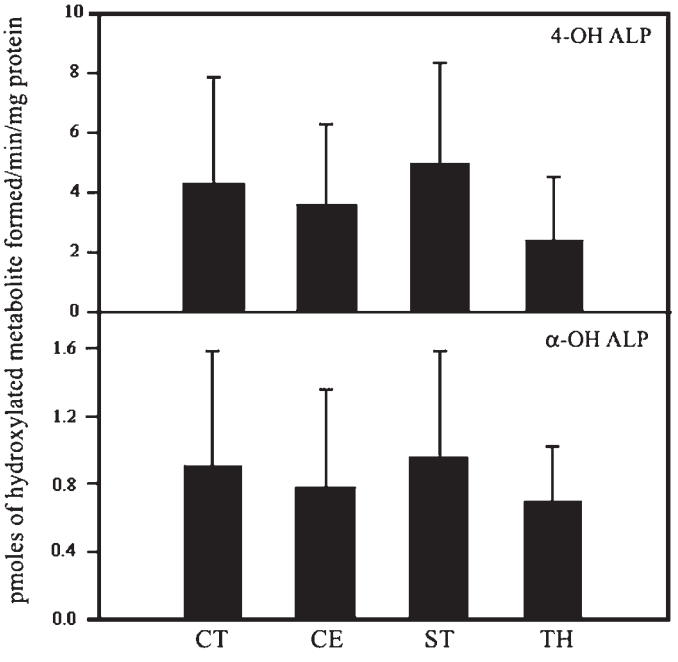

Figure 1 Metabolism of ALP to 4-OHALP and α-OHALP by human brain microsomes. Microsomes (1 mg protein ml^{-1}) prepared from brain regions of human autopsy tissue were incubated with 1mM ALP for 15 min in presence of NADPH. The amount of 4-OHALP and α-OHALP formed were estimated. Values are average of duplicate analysis of brain regions obtained from four individuals. Data is presented as mean ± SD (n = 4 subjects). The brain regions are represented as CT-cortex, CE-cerebellum, ST-striatum, TH-thalamus.

Inhibition of Alprazolam Hydroxylase Activity in Rat and Human Brain Microsomes

We incubated microsomes from rat (Figure 2a) and human brain cortex (Figure 2b) with the common inhibitor of P450, piperonyl butoxide prior to estimation of enzyme activity. This pretreatment resulted in the inhibition of alprazolam hydroxylase activities by 60% of 4-hydroxy alprazolam and 85% of α-hydroxy alprazolam in rat brain as compared to control and by 77% of 4-hydroxy alprazolam and 70% of α-hydroxy alprazolam as compared to controls in human brain, indicating that the metabolism was indeed mediated by P450. Preincubation with quinidine, an inhibitor of P4502D had a substantially lower inhibitory effect on the hydroxylase activities as compared to ketoconazole (a specific inhibitor of P4503A). Further, preincubation of the rat brain microsomes with the antiserum to P4503A resulted in the inhibition of alprazolam hydroxylase activities by 89% of 4-hydroxy alprazolam and 86% of α-hydroxy alprazolam as compared to non-immune serum, indicating that the biotransformation were indeed mediated by P4503A.

Relative Rates of Formation of 4-Hydroxy and α-Hydroxy Alprazolam by Rat and Human Brain Microsomes

We measured the rate of formation of 4-hydroxy and α-hydroxy alprazolam by rat liver and brain microsomes. In rat liver, the rates of formation of 4-hydroxy and α-hydroxy alprazolam were 167.7 and 3.2 pmol min^{-1} mg^{-1} protein, respectively. Thus, the rate of formation of α-hydroxy alprazolam is about 1.9% of the total 4-hydroxy alprazolam formed after 15 min of microsomal incubation in presence of NADPH. In brain, the rates of formation of 4-hydroxy and α-hydroxy alprazolam were 5.44 and 2.42 pmol min^{-1} mg^{-1} protein, respectively, thus indicating that the amount of α-hydroxy alprazolam formed was 44.5% of the total 4-hydroxy alprazolam after similar incubation period of 15 min (Figure 3a). When we looked at the relative rates of formation of the hydroxylated metabolites after shorter incubation periods, such as 5–10 min, we found that the amount of α-hydroxy alprazolam formed in brain microsomes after 5 min of incubation was 35% more than the detectable amount of 4-hydroxy alprazolam indicating that α-hydroxy alprazolam which is known to be relatively unstable, was undergoing degradation during longer incubation periods. Thus, α-hydroxy alprazolam (which is pharmacologically more active) formed in greater amounts in the brain as compared to liver, but since this metabolite has a tendency to degrade during microsomal incubation relatively lesser amounts are detected during longer microsomal incubations. α-Hydroxy alprazolam was also formed in relatively larger amounts in human brain microsomal incubations (Figure 3b).

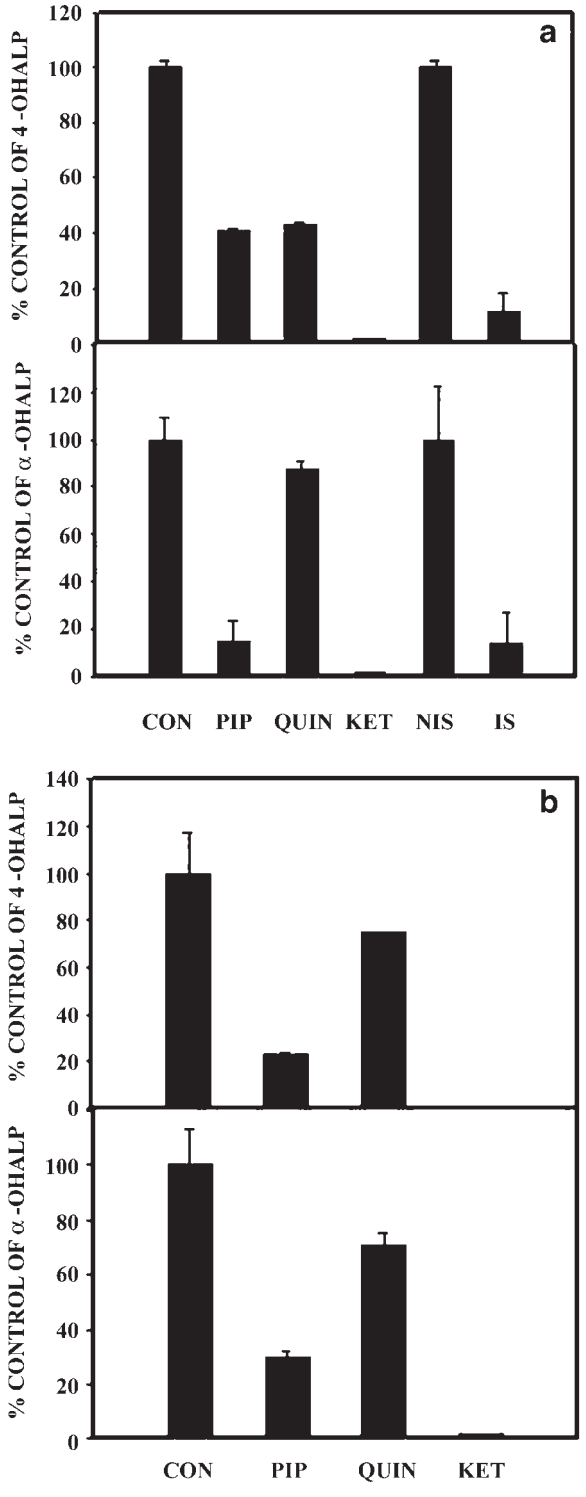

Figure 2 Effect of inhibitors and antiserum to P4503A4 on the formation of 4-OHALP and α-OHALP by rat (a) and human (b) brain microsomes. Microsomal incubations were carried out in presence of PIP (piperonyl butoxide 0.5 mM), QUIN (quinidine 1mM), KET (ketoconazole 5 μM), or CON (in absence of any of the above) and NIS (nonimmune serum 50 μl) or IS (immune serum 50 μl). Data represents the amount of metabolites formed min^{-1} mg^{-1} protein as percent of the control values (CON). Values are (a) mean ± SD from three different batches of rat brain microsomes and (b) mean ± SD (n = 4 subjects).

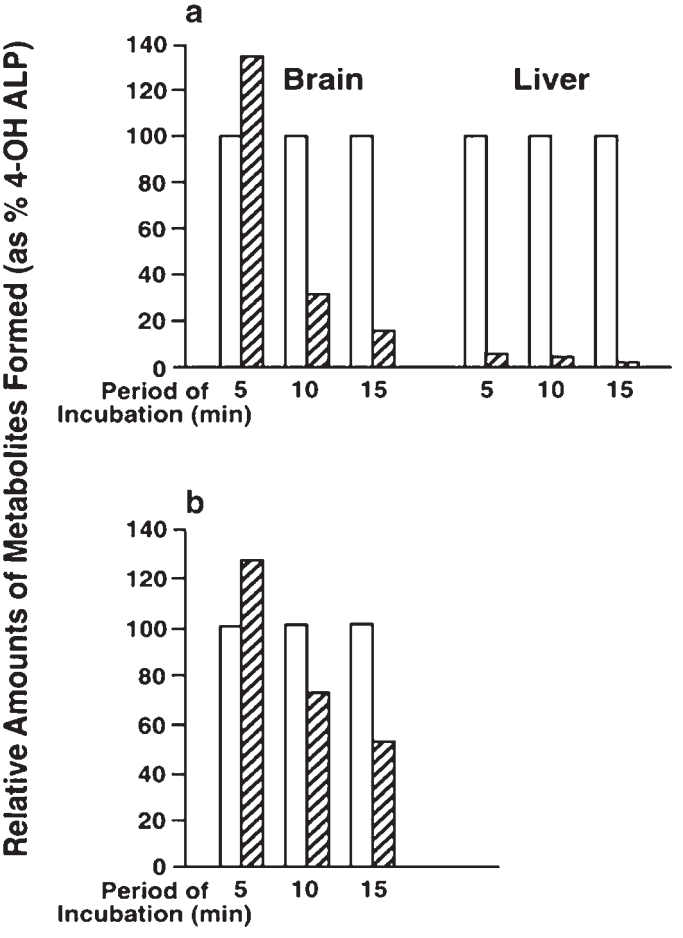

Figure 3 Relative rates of formation of α-OHALP and 4-OHALP from ALP by rat (a) brain and liver and human brain (b) microsomes. Relative amounts of the hydroxylated metabolites formed are expressed as percent of 4-OHALP formed at varying time periods. Empty bars represent 4-OHALP and filled bars represents α-OHALP. The values are average of three analysis. The rates of formation of 4-hydroxy alprazolam and α-hydroxy alprazolam in liver after 15 min of microsomal incubation were 167.7 and 3.23 pmoles of product formed min^{-1} mg^{-1} protein, respectively. In rat brain, under similar conditions, the rates were 5.44 and 2.4 pmoles of hydroxylated metabolites formed, while in the human brain the rates were 2.03 and 1.15 pmoles of product formed min^{-1} mg^{-1} protein.

Northern Blot Analysis of CYP3A Expression in Rat Brain

Northern blot analysis of total RNA from rat and human brain cortex demonstrated the expression of transcripts belonging to CYP3A subfamily. The molecular mass of the transcript was approximately 1.6 Kb (Figure 4).

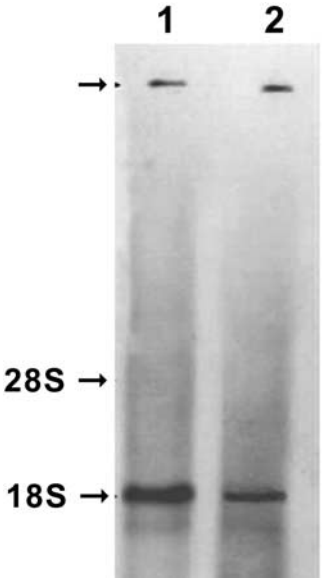

Figure 4 Northern blot analysis showing the constitutive expression of CYP3A mRNA in rat and human brain. Total RNA from rat brain cortex (lane 1, 12 μg) and human brain cortex (lane 2, 6 μg) were electrophoresed under denaturing conditions. After transfer to nylon membrane, the blots were hybridized with antisense riboprobe prepared using cDNA to CYP3A1. The mobility of the 18S and 28S ribosomal RNA is indicated. The CYP3A mRNA in rat and human brain was seen as a band at approximately 1.6 Kb.

Localization of CYP3A mRNA in Rat Brain by Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) studies demonstrated the presence of mRNA belonging to CYP3A subfamily predominantly in neuronal cells in rat brain regions. The neuronal cells in the cerebral cortex showed intense cytosolic staining, indicating the presence of the CYP3A mRNA (Figure 5a), while the sections hybridized with the sense probe showed no fluorescence (Figure 5b). In the cerebellum, the Purkinje cells showed intense fluorescence, while the granule cell layer was relatively less intensely stained (Figure 5c). Intense fluorescence was seen in hippocampus, in the pyramidal neurons of CA1, CA2 and CA3, and in the granule cell layer in the dentate gyrus (Figure 5e), while similar sections hybridized with the sense digoxigenin labeled probe showed no fluorescence (Figure 5f). Neurons in the thalamus were also stained indicating the presence of CYP3A (Figure 6a). In the mid-brain, the reticular neurons were selectively labeled, indicating the predominant presence of CYP3A mRNA in these cell populations (Figure 6b). In the cervical region of the spinal cord, the neurons were intensely stained including the large anterior horn cell (Figure 6c and d).

Localization of CYP3A mRNA in Human Brain by Fluorescence *in situ* Hybridization

Fluorescence *in situ* hybridization (FISH) studies demonstrated the presence of CYP3A mRNA predominantly in neuronal cells in human brain regions. High levels of CYP3A mRNA was seen in neuronal cells in the cerebral cortex (Figure 7a), while sections hybridized with the sense probe showed no fluorescence (Figure 7b). In the cerebellum, Purkinje cells showed intense fluorescence while the granule cell layer was less stained (Figure 7c). The reticular neurons in mid-brain were also labeled indicating the presence of CYP3A mRNA (Figure 7e). Intense fluorescence was seen in hippocampus (Figure 8), in the pyramidal neurons of CA1, CA2 and CA3 subfields and in the granule cell layer in the dentate gyrus, while similar sections hybridized with the sense digoxigenin labeled probe showed no fluorescence (Figure 8d). The pyramidal neurons in CA1 showed intense cytosolic staining (Figure 8c). The neurons in CA2 were also labeled and the compact CA2 neuronal cell layer was clearly discernable (Figure 8a). In CA3, pyramidal neurons were intensely fluorescent (Figure 8b), along with the interneurons of the hilius. The granule cell layer of the dentate gyrus was also intensely stained (Figure 8b) indicating the presence of high amounts of CYP3A mRNA, similar to that seen in the rat brain.

Immunoblot Analysis of Microsomes from Rat and Human Brain Using Antiserum to Hepatic P4503A

Immunoblot analysis of rat brain microsomal proteins using antiserum to the hepatic P4503A4 showed the constitutive presence of P4503A in the brain regions of untreated rat brain (Figure 9a). Similar immunoblot studies were carried out using microsomes prepared from human brain regions obtained at autopsy (Figure 9b). The constitutive presence of P4503A was detectable in all the brain regions examined in both rat and human brain. An immunoreactive protein of molecular weight approximately 52.8 kDa was detectable in microsomes prepared from various regions of human brains such as cortex, cerebellum, striatum and hippocampus (Figure 9).

Immunohistochemical Localization of P4503A in Rat Brain

Immunohistochemical studies demonstrated the presence of P4503A protein predominantly in neuronal cells in rat brain. Intense immunostaining was observed in neurons in rat brain cortex indicating the presence of P4503A (Figure 10d). At higher magnification (Figure 10e) immunostaining of apical dendrites was also observed. No immunostaining was seen in control sections pretreated with non-immune serum (Figure 10f). Intense immunostaining of neurons in olfactory bulb was seen, indicating presence of P4503A (Figure 10a). The neurons in the olfactory glomeruli were intensely stained (Figure 10b). No staining was seen in external plexiform layer (EPL) while the granule cell layer (GL) was intensely stained (Figure 10a). Significant immunostaining of cerebellar granular cell layer (GL) and sparse staining of the cells in the molecular layer (ML) was observed (Figure 11a1). Immunostaining was seen in CA1, CA2 and CA3 subfields of hippocampus (Figure 11d1) and granular cell layer of the dentate gyrus (arrow in Figure 11e1) was intensely stained. The interneurons of the hilius and CA3 pyramidal neurons were also immunostained (Figure 11e1).

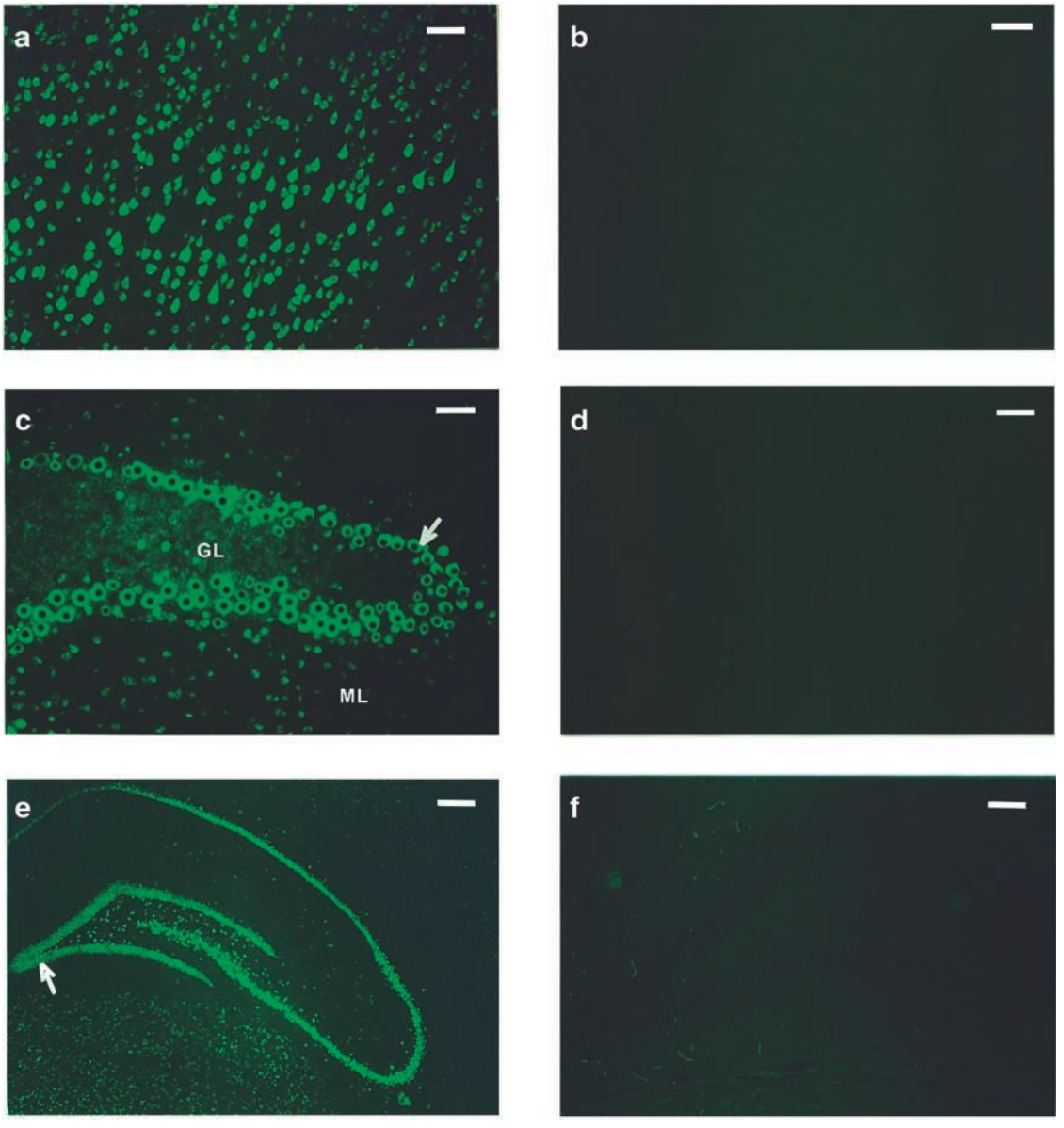

Figure 5 Localization of CYP3A mRNA in rat brain using fluorescence *in situ* hybridization. (a) The presence of CYP3A in the neurons of cerebral cortex (arrow); Bar = 50 μm. (b) Control section hybridized with the sense probe; Bar = 100 μm. (c) Fluorescent labelling of the Purkinje cells (arrow), in the rat cerebellum; Bar = 50 μm. Control section of rat cerebellum hybridized with sense probe is depicted in (d); Bar = 100 μm. (e) Intense fluorescence was seen in granule cell layer of the dentate gyrus (arrow). The CA1, CA2 and CA3 subfields of the hippocampus was also intensely fluorescent; Bar = 200 μm. (f) Control sections hybridized with sense probe showed no fluorescence; Bar = 100 μm.

Immunostaining of striatal neurons was observed indicating the presence of P4503A protein (Figure 12a2 and b2). Expression of P4503A was also seen in midbrain region of rat brain (Figure 12f2). P4503A expression was also seen in the neurons of the anteroventral nucleus of thalamus (Figure 12e2). Intense staining was seen in the island of Calleja (arrow in Figure 12c2) indicating the presence of P4503A in these regions. Staining of glial cells in the corpus callosum was also observed (Figure 12d2).

Localization of P4503A by Immunofluorescence

Immunofluorescence analysis using antiserum to liver P4503A demonstrated the predominant presence of P4503A in neuronal population in concordance with the *in situ* experiments. In the cerebellum, granule cells and Purkinje cells were stained (Figure 13a), with the Purkinje cells being more intensely stained compared to granule cells, similar to the observation made in the *in situ* studies. Fluorescent staining of the neuronal cell body and apical dendrites was

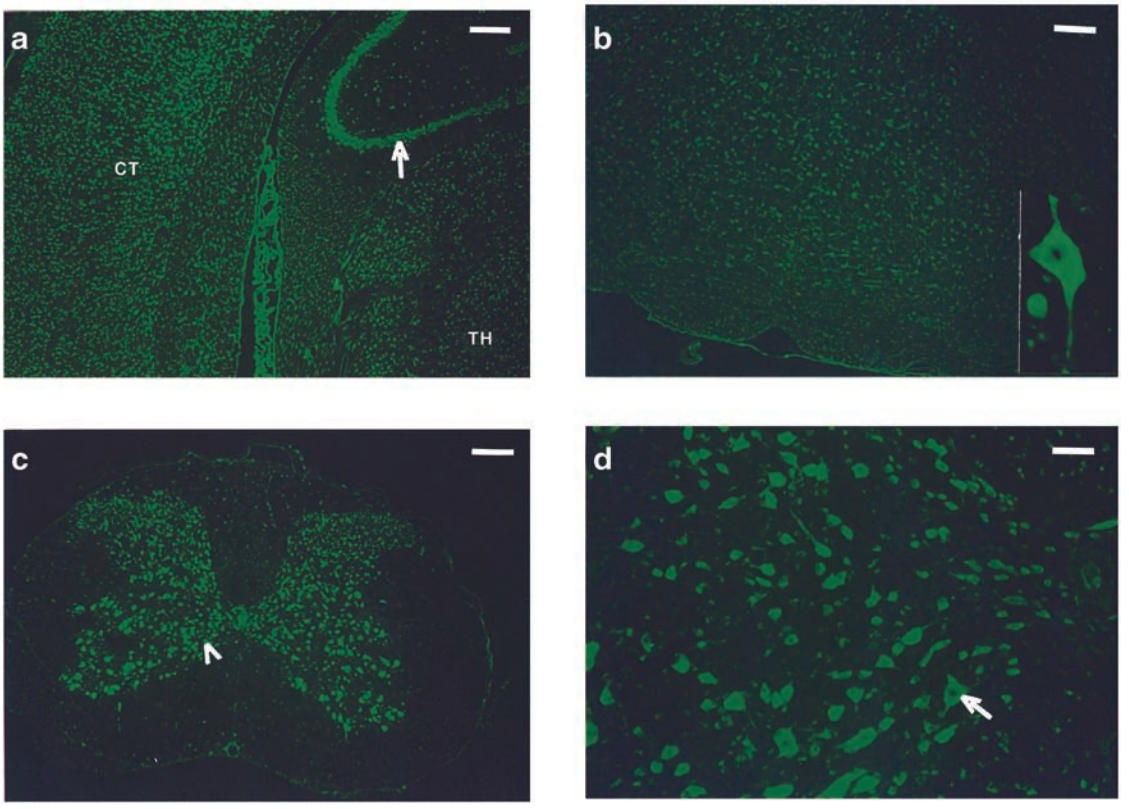

Figure 6 Localization of CYP3A mRNA in control rat brain using fluorescence *in situ* hybridization. (a) CYP3A mRNA was observed in the ventral nucleus of the thalamus and in the fimbria of the hippocampus (arrow); Bar = 200 μm. Inset: higher magnification of a giant reticular neuron. (b) The intense fluorescence of reticular neurons in the midbrain expressed the CYP3A mRNA; Bar = 200 μm. (c) Intense fluorescence was seen in the spinal cord section; Bar = 200 μm. (d) The anterior horn cells of the spinal cord (arrow) were intensely fluorescent indicating the substantial expression of CYP3A; Bar = 50 μm.

seen in frontal cortex (Figure 13d). The CA1 neurons in the hippocampus were also stained (Figure 14a and b) where immunostaining was also observed in the synaptic terminals. Reticular neurons of the midbrain (Figure 14d and e) were also immunostained. Similar sections incubated with non-immune serum did not show any staining (Figure 14f).

DISCUSSION

Hydroxylation of drugs by hepatic P450 results in the formation of hydrophilic metabolites, which can be easily excreted by the kidney. However, if such hydroxylated metabolites were to be formed in the brain it would result in their prolonged presence due to the presence of the blood-CSF barrier. The two hydroxylated metabolites of alprazolam are known to have differential pharmacological effects. α-Hydroxy alprazolam has about 77% of the intrinsic pharmacological activity of the parent drug, while 4-hydroxy alprazolam has only about 14% of the activity.[14] However, since the plasma levels of both the hydroxylated metabolites are considerably lower than the parent drug in humans,[15] it has been concluded that they do not play any significant role in the anti-anxiety effects of alprazolam, which are mediated in the brain. This study presents the first evidence for the formation of the hydroxylated metabolites of alprazolam *in situ*, at the site of action, in the brain where they can exert pharmacological action regardless of the plasma concentration. This is of significance since the hydroxylated metabolites would potentially have a prolonged presence and lowered clearance from brain.[10] Further, since the isoform of P450 belonging to the P4503A sub-family which mediates alprazolam metabolism in the brain is localized predominantly in neuronal cell population (Figure 5), the site of action of these drugs, the intrinsic concentration of these metabolites in the neuronal cells may be significant enough to play a role in the local pharmacological action of the drug. Further, the activity measurements reported in the present study have been ascertained using microsomes prepared from the brain tissue, which comprises of both glial and neuronal cells. The measured activity would be the average of the contributions by the neuronal and glial cells, thus under-estimating the biotransformation in the neurons. P4503A is localized in the neurons in the cortex, cerebellum, olfactory tubercle and hippocampus of rat brain which co-localizes with the benzodiazepine binding sites in $GABA_A$ receptor, such as, neuronal cell layers in cerebral cortex, granule cell layers of cerebellum, dentate gyrus and pyramidal cell layers (CA1, CA2 and CA3) of hippocampus, olfactory tubercle, island of Calleja and thala-

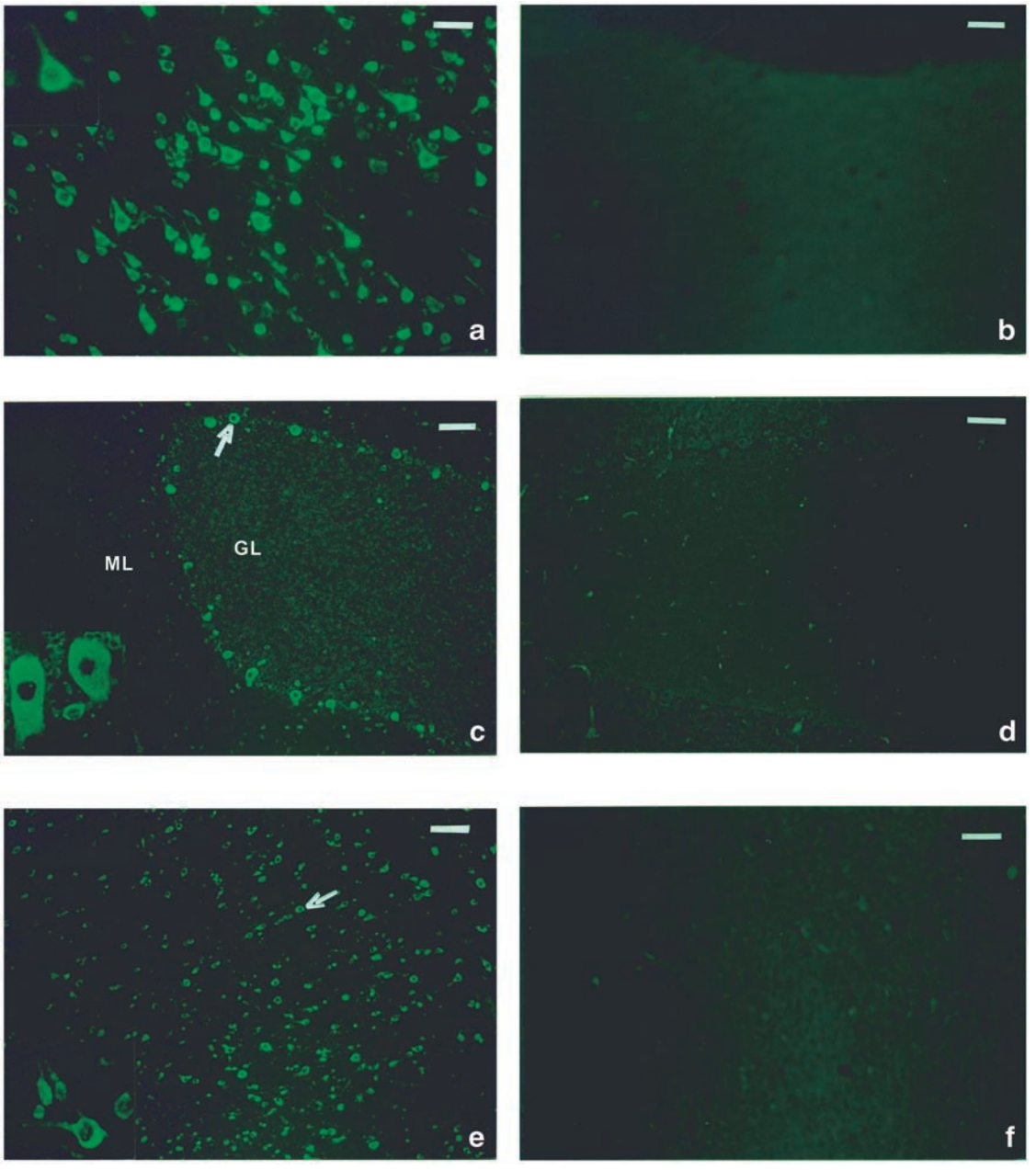

Figure 7 Localization of CYP3A mRNA in human brain by fluorescent *in situ* hybridization. (a) Intense fluorescence was seen in the neuronal cell layers of the human brain cortex; Scale bar = 200 μm. Inset: higher magnification of a fluorescent cortical neuron expressing CYP3A mRNA. (b) Control section hybridized with sense probe did not show any fluorescence; Scale bar = 100 μm. (c) Fluorescent labeling of the Purkinje cells (arrow) and the granule cell layer (GL) was seen in the human cerebellum. Sparse staining of the cells in the molecular layer (ML) was also noted; Scale bar = 50 μm. Inset: higher magnification of the Purkinje neurons. Control section of the human cerebellum hybridized with the sense probe is depicted in (d); Scale bar = 100 μm. (e) The reticular neurons in the midbrain expressed CYP3A mRNA; Scale bar = 50 μm. Inset: higher magnification of a reticular neuron. (f) Control section of midbrain hybridized with the sense probe; Scale bar = 100 μm.

mus[16] indicating that the metabolism of alprazolam to its pharmacologically active metabolite could occur at the site of drug action.

Another important finding in the present study, being reported for the first time, is the intrinsic difference in the metabolite profile seen in brain and liver. While it has been observed that the P450 levels in the brain are one-tenth to one-fifteenth of that typically seen in liver and it is generally acknowledged that the capability of the brain to effect P450 mediated metabolism of drugs is also substantially lower, the observations made in this study demonstrate that the above considerations not withstanding, pharmacologically

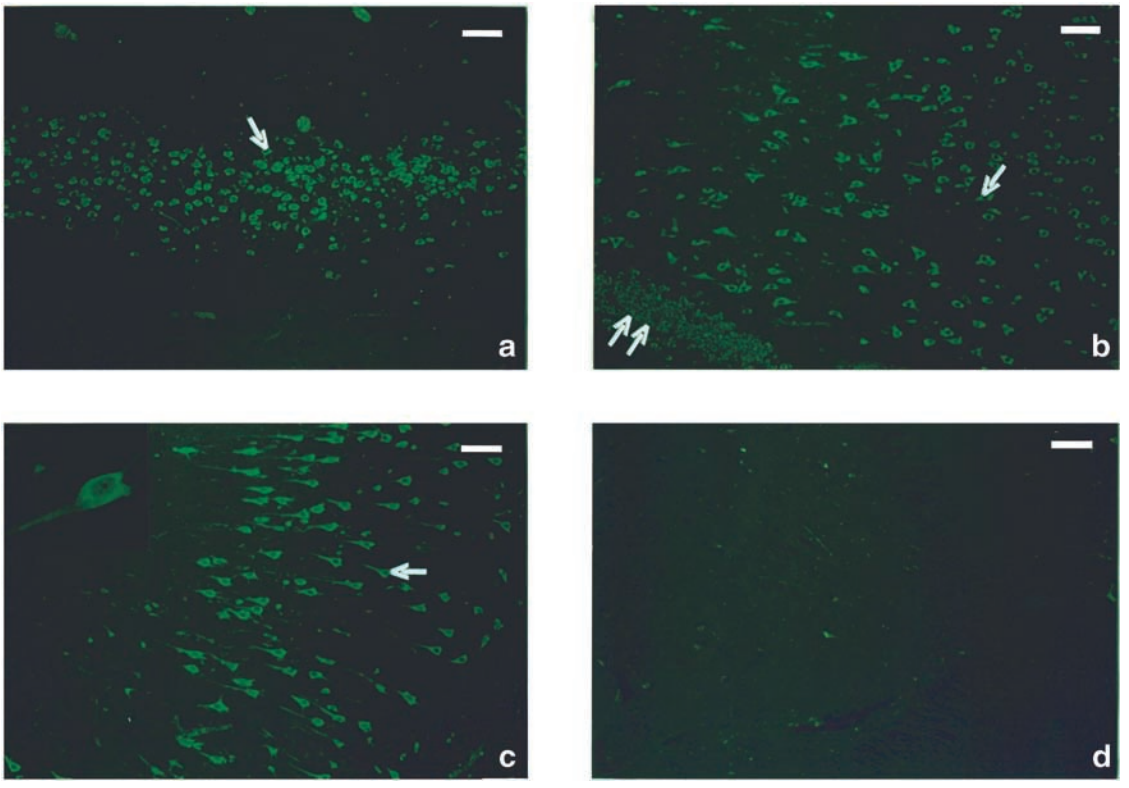

Figure 8 Localization of CYP3A mRNA in human brain by fluorescent *in situ* hybridization. (a) The presence of CYP3A mRNA in the pyramidal neurons of CA2 subfield in the hippocampus (arrow) is depicted; Scale bar = 200 μm. (b) A view of fluorescent granule cell layer of the dentate gyrus (double arrow) is presented, intense fluorescence of the hilial neurons (arrow) and CA3 pyramidal neurons is also seen; Scale bar = 200 μm. (c) The presence of CYP3A mRNA was observed in CA1 neurons (arrow) which were intensely fluorescent; Scale bar = 200 μm. Inset: higher magnification of CA1 neuron. (d) Control section hybridized with sense probe did not exhibit any fluorescence; Scale bar = 100 μm.

active metabolites can be formed in significant amounts in the brain. Thus, in rat and human brain, α-hydroxy alprazolam is formed in relatively higher amounts than in the liver. While, the amount of 4-hydroxy alprazolam formed in rat brain is 3.2% of the concentration in liver, α-hydroxy alprazolam concentration is 75% of the corresponding level in liver. Further, in the liver 4-hydroxy alprazolam is the major metabolite and concentration of the α-hydroxy metabolite is only 1.9% of the corresponding 4-hydroxy alprazolam levels. However, in the brain, after 15 min microsomal incubation, we find that the relative concentration of α-hydroxy metabolite is 44.5% of the 4-hydroxy metabolite. α-Hydroxy alprazolam is known to be unstable under experimental conditions, therefore we measured the relative amounts of metabolites formed after shorter incubation periods (Figure 3). α-Hydroxy alprazolam is formed in greater amounts than 4-hydroxy alprazolam in rat brain microsomal incubation, while this is not reflected in experiments using rat liver microsomes.

The studies carried out using rat brain microsomes were replicated using human autopsy brain tissue obtained from traffic accident victims. Our earlier studies have shown that the brain P450 levels and NADPH cytochrome c reductase activity (an essential component of P450 mono-oxygenase system) are preserved in brain up to 12 h of post-mortem delay.[17] The tissues used in these studies were obtained within this time period.

Thus, the present study demonstrates the capability of the human brain to metabolize alprazolam to its hydroxylated metabolites, but more importantly, in a manner similar to that seen in rat brain, α-hydroxy alprazolam, the pharmacologically more active metabolite, was formed in relatively greater amounts as compared to 4-hydroxy alprazolam. Our results are in agreement with the studies of Banks *et al*[18] where a single dose of ^{14}C-alprazolam was administered to rats and the levels of the parent drug and the hydroxylated metabolites were measured in plasma and brain. They observed that the plasma concentration of α-hydroxy alprazolam (13% of 4-hydroxy alprazolam concentration) was quite low, while in the brain although the absolute concentration of the hydroxylated metabolites was low, α-hydroxy alprazolam concentration was 266% of 4-hydroxy alprazolam.

The salient features of the current study are that it presents conclusive evidence for differences in the metabolism of drugs in liver, the major organ involved in biotransformation of drugs and the brain, the site of action of a variety of psychoactive drugs which are used extensively

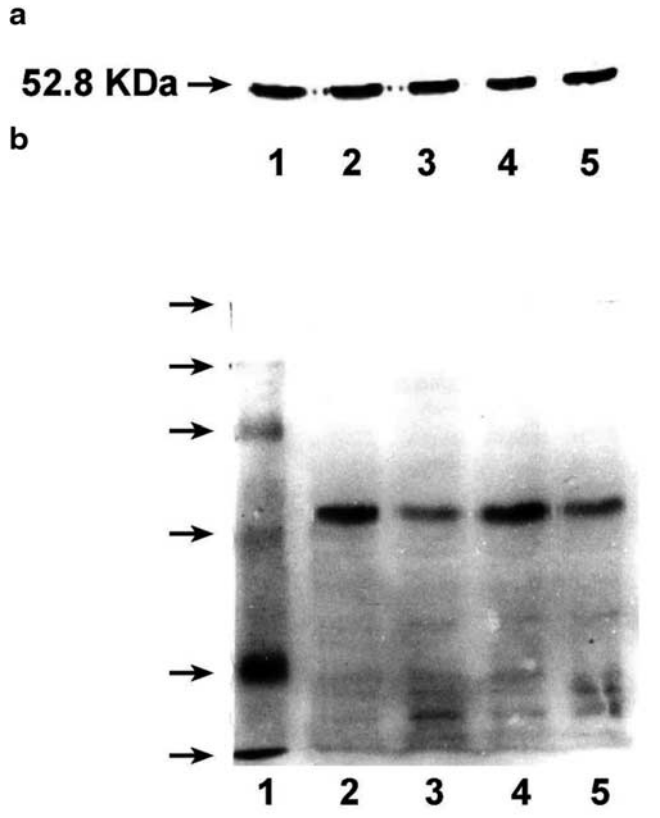

Figure 9 Immunoblot analysis of microsomal protein from rat (A) and human (B) brain regions stained with antiserum to hepatic P4503A. Microsomal protein (50 μg) prepared from rat (a) and human (b) brain regions were subjected to SDS–PAGE followed by immunoblotting onto nitrocellulose membrane with antiserum to P4503A4. Immunostained bands were detected at 52.8 KDa. (a) The lanes contained microsomal protein from rat brain cerebral cortex (lane 1), cerebellum (lane 2), midbrain (lane 3), hippocampus (lane 4) and thalamus (lane 5). (b) Immunostained bands were also detectable in microsomes from human brain regions such as cortex (lane 2), cerebellum (lane 3), striatum (lane 4) and hippocampus (lane 5) from subject II. Lane 1 was loaded with molecular weight markers indicated by arrows (top to bottom—198, 113, 75, 48.9, 17.4 and 6.8 KDa, respectively).

in the treatment of mental illnesses and often for prolonged periods of time. The striking differences seen in the metabolism of alprazolam in liver and brain, and more importantly the formation of greater amounts of α-hydroxy alprazolam, the pharmacologically more active metabolite, points to the need for examining the metabolism of drugs at their site of action rather than taking hepatic metabolism alone into account. This is also important because brain P450 forms are different from liver; for example P4503A1 is induced by phenobarbital in rat liver but not in brain.[19] These are relevant not only for known drugs, but also newer drugs which are in the discovery and development stage. The similarities seen in the metabolic capability between rat and human brain indicate that experiments using rat brain microsomes can be used as a pointer to understand potential metabolism in human brain tissue.

In conclusion, the present study demonstrates the significant differences that exist between hepatic and cerebral metabolism of drugs such as alprazolam and suggest the importance of understanding biotransformation pathways in brain. Of particular importance are the observations made using human brain tissue, which not only provide the first evidence for metabolism of psychoactive drugs in human brain, but also demonstrate the constitutive expression of the enzyme involved in the biotransformation and its localization in the neuronal cells, the site of action of these drugs.

MATERIALS AND METHODS

Materials

Alprazolam and its two principal metabolites, 4-OHALP and α-OHALP were obtained as gifts from Upjohn Company, Kalamazoo, MI, USA. Dithiothreitol, phenylmethyl sulfonyl fluoride, aprotinin, leupeptin and NADPH were purchased from Sigma Chemical, USA. Northern blot analysis was performed using the digoxigenin labeling kit from Boehringer Mannheim, Germany. Fluorescent *in situ* hybridization experiments were performed using the tyramide signal–amplification kit from NEN Life Sciences Products, USA.

Animals

Male Wistar rats (3–4 months, 225–250 g) were obtained from the Central Animal Research Facility of the National Institute of Mental Health and Neurosciences. Animals had access to a pelleted diet (Lipton India, Calcutta, India) and water ad libitium. All animal experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the institutional ethics committee. All efforts were made to minimize animal suffering, to reduce number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

Human Brain Tissue

Human brain, from traffic accident victims was obtained from the Human Brain Tissue Repository of the Institute. Both male and female brains were used. The age and gender of the subjects are: (subject I) 51 year old female (postmortem delay 10 h); (subject II) 32 year old male (postmortem delay 4 h); (subject III) 8 year old female (postmortem delay 8 h); (subject IV) 65 year old male (postmortem delay 6 h); and (subject V) 21 year old male (postmortem delay 1.5 h). After autopsy, the brains were washed in ice-cold saline and dissected into different regions such as cortex, cerebellum, hippocampus, striatum, midbrain and thalamus based on standard anatomical markings. All regions were frozen and stored at $-70^{\circ}C$ immediately. These tissues were thawed on ice prior to the preparation of microsomes as described below.

Preparation of Microsomes

Animals were anesthetized with ether and perfused transcardially with ice-cold Tris buffer (100 mM, pH 7.4) containing KCl (1.15% w/v) prior to decapitation and removal of brain.

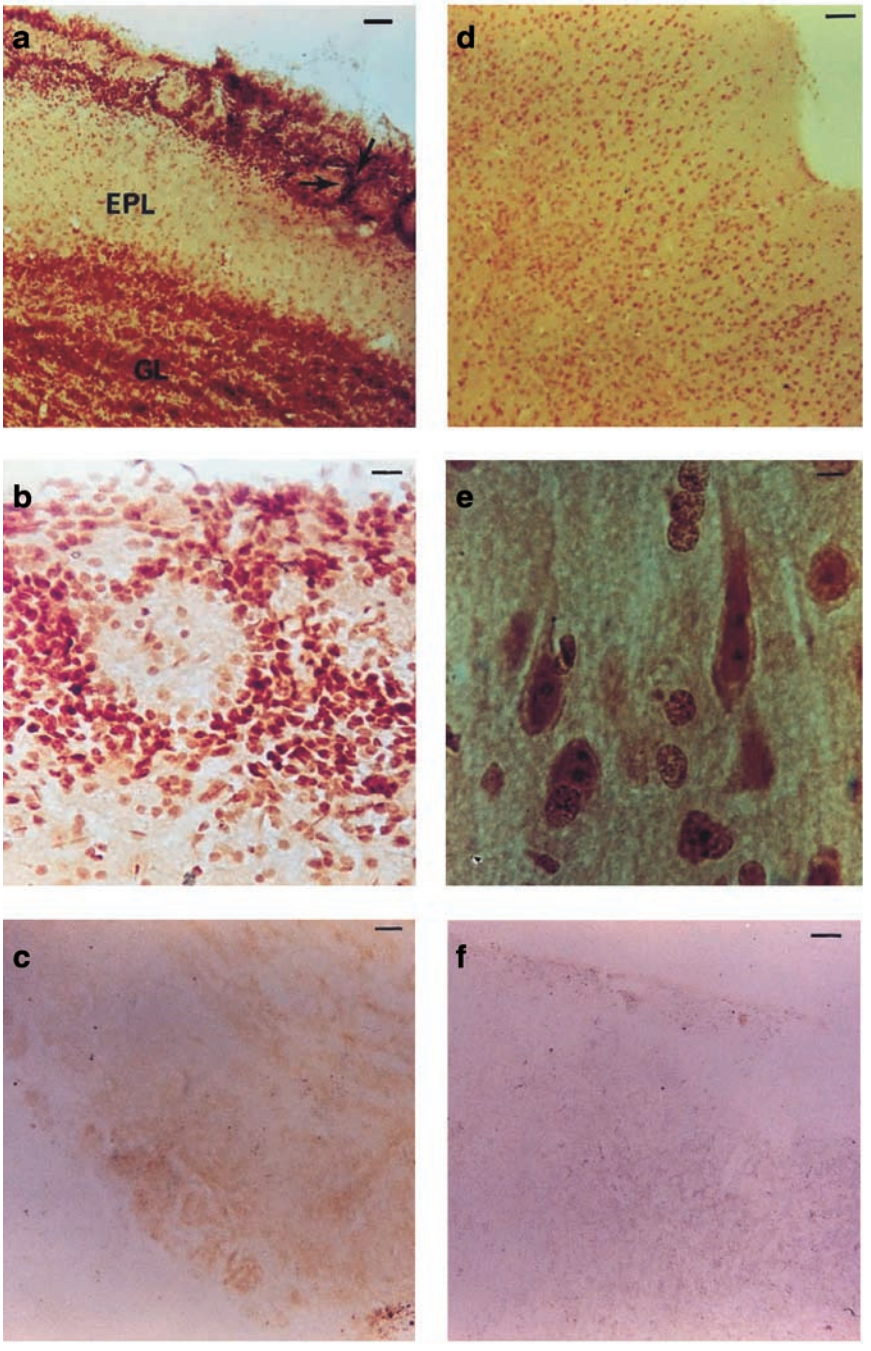

Figure 10 Localization of P4503A in rat brain by immunohistochemical analyses. (a) Intense immunolabelling of neurons in olfactory bulb was seen, indicating presence of P4503A. The neurons in the glomeruli (arrow) were intensely stained. No staining was seen in external plexiform layer (EPL) while the granule cell layer (GL) was intensely stained; Bar = 100 μm. (b). Higher magnification of the glomeruli in the olfactory bulb; Bar = 25 μm. (c) Control section incubated with non-immune serum did not show immunostaining in the olfactory bulb; Bar = 100 μm. (d) Rat brain section showing intense immunostaining of cortical neurons indicating the presence of P4503A; Bar = 100 μm. (e) Higher magnification of cortical neurons showing intense immunostaining; Bar = 10 μm. (f) Control section pretreated with non-immune serum did not show immunostaining in the cerebral cortex; Bar = 100 μm.

Whole brain from rats (tissue pooled from 8–10 rats) or human brain regions or rat brain regions (cortex, cerebellum, mid-brain, hippocampus and thalamus) were dissected out using standard anatomical landmarks.[20] The tissues were homogenized using a Potter–Elvehjem homogenizer in nine volumes of ice-cold Tris buffer (0.1 mM), EDTA (0.1 mM), KCl (1.15% w/v), phenyl methyl sulfonyl fluoride (0.1 mM), butylated hydroxytoluene (22 μM), glycerol (20%, v/v),

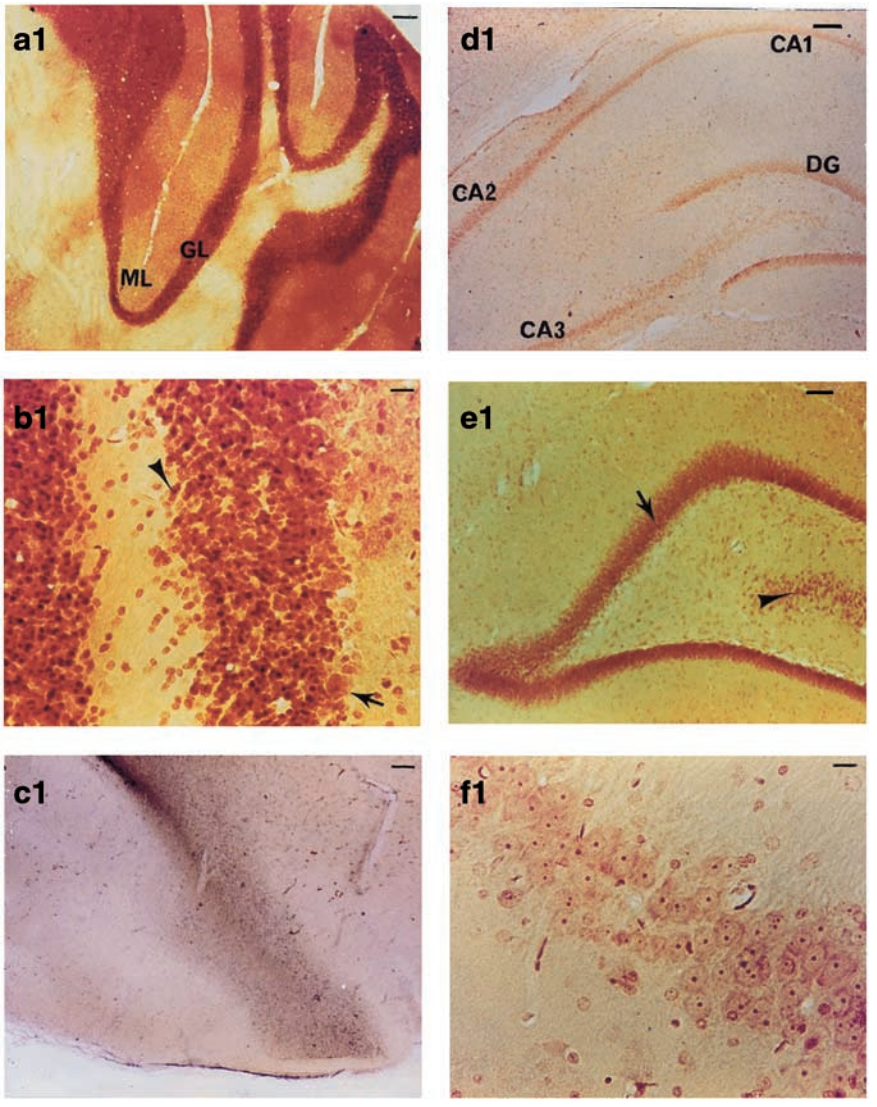

Figure 11 Localization of P4503A in rat brain by immuno-histochemical analyses. (a1) Intense immunostaining of granular cell layer (GL) of cerebellum was seen, while the molecular layer (ML) was sparsely stained; Bar = 200 μm. (b1) Higher magnification of cerebellum showing intense staining of the granule cell layer (arrow head) and Purkinje cells (arrow) indicating the presence of P4503A; Bar = 25 μm. (c1) No staining was seen in cerebellum section treated with non-immune serum; Bar = 200 μm. (d1) Intense immunostaining of P4503A in dentate gyrus (DG), CA1, CA2 and CA3 subfields of hippocampus; Bar = 200 μm. (e1) Higher magnification of the granular cell layer of the dentate gyrus (arrow) showing intense immunostaining for P4503A. Staining of the interneurons of the hilius (arrowhead) was also observed; Bar = 50 μm. (f1) Higher magnification of the CA1 pyramidal neurons showing the presence of P4503A; Bar = 25 μm.

aprotinin (0.001%, w/v) and leupeptin (0.001%, w/v), previously bubbled with nitrogen (buffer A). The homogenate was centrifuged at 17 000 g for 30 min at 4°C. Thereafter, the supernatant was centrifuged at 100 000 g for 1 h to give the microsomal pellet. The pellet was suspended in a small volume of buffer A, aliquoted, flash–frozen in liquid nitrogen and stored at −70°C. The protein concentration was measured by dye-binding method.[21]

HPLC Assay of Alprazolam

Brain microsomal incubation (2 ml) was performed in Tris–HCl buffer (50mM, pH 7.4) containing $MgCl_2$ (20 mM), microsomal protein (0.5 mg–2 mg ml^{-2} of incubation) and alprazolam (0.25–1.5 mM). The reaction was initiated by addition of NADPH (1 mM final concentration) after preincubation at 37°C for 4 min. Blanks containing no NADPH or substrate were also run simultaneously. The reaction was terminated after 15–60 min by the addition of NH_4OH (0.1%, v/v) and 5 ml of dichloromethane. The microsomal incubations were vortexed, centrifuged at 4000 g (5 min) and the lower organic phase was pooled and evaporated under nitrogen atmosphere at 45°C . The residue was dissolved in 0.4 ml of the HPLC mobile phase consisting of potassium phosphate buffer (50 mM, pH 9) containing ace-

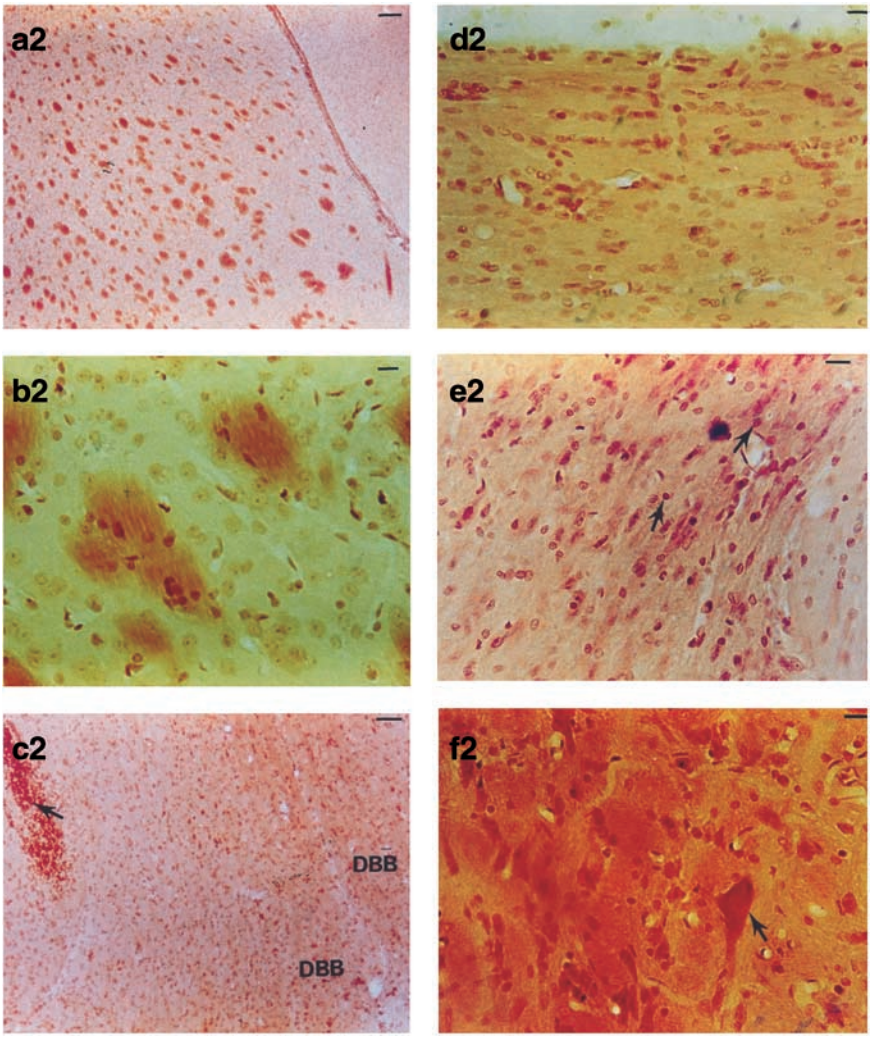

Figure 12 Localization of P4503A in rat brain by immunohistochemical analyses. (a2) Immunostaining of striatal neurons indicating the presence of P4503A; Bar = 200 μm. (b2) Higher magnification of the striatal neurons showing the presence of P4503A; Bar = 25 μm. (c2) Intense staining was seen in island of Calleja (arrow) and in the diagonal band of Broca (DBB) indicating the presence of P4503A; Bar = 25 μm. (d2) Staining of cells was also seen in corpus callosum; Bar = 25 μm. (e2) The neurons in the anteroventral nucleus of the thalamus were immunostained (arrows); Bar = 25 μm. (f2) Immunostaining of reticular neuron (arrow) in the midbrain indicating the presence of P4503A; Bar = 25 μm.

tonitrile (30% v/v), filtered and injected onto an HPLC column consisting of SUPELCOSIL (LC-18 column) at a flow rate of 1 ml min^{-1}. The formation of metabolites (4-OHALP and α-OHALP) was detected by monitoring the UV absorption at 224 nm. The amount of metabolites formed was estimated from the standard curve. All analysis was performed in duplicates.

Inhibition Experiments

Immunoinhibition was performed by preincubation of rat brain and human cortical microsomes with varying concentrations of non-immune serum or antiserum to rat liver P4503A4 for 30 min at 37°C prior to the assay of alprazolam metabolism. Chemical inhibition was performed by preincubation of microsomes with piperonyl butoxide (0.5 mM final concentration), quinidine (1 mM final concentration) or ketoconazole (5 μM final concentration) for 30 min. at 37°C prior to the addition of substrate and NADPH for microsomal incubation.

Immunoblot Analyses

Microsomal proteins from rat (50 μg) and human (50 μg) brain regions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[22] Proteins were transferred from the gel to nitrocellulose membranes.[23] The membranes were immunostained with antiserum to liver P4503A4, followed by incubation with anti rabbit IgG labeled with alkaline phosphatase (Vector Laboratories, USA.). The immunostained bands were detected using nitroblue

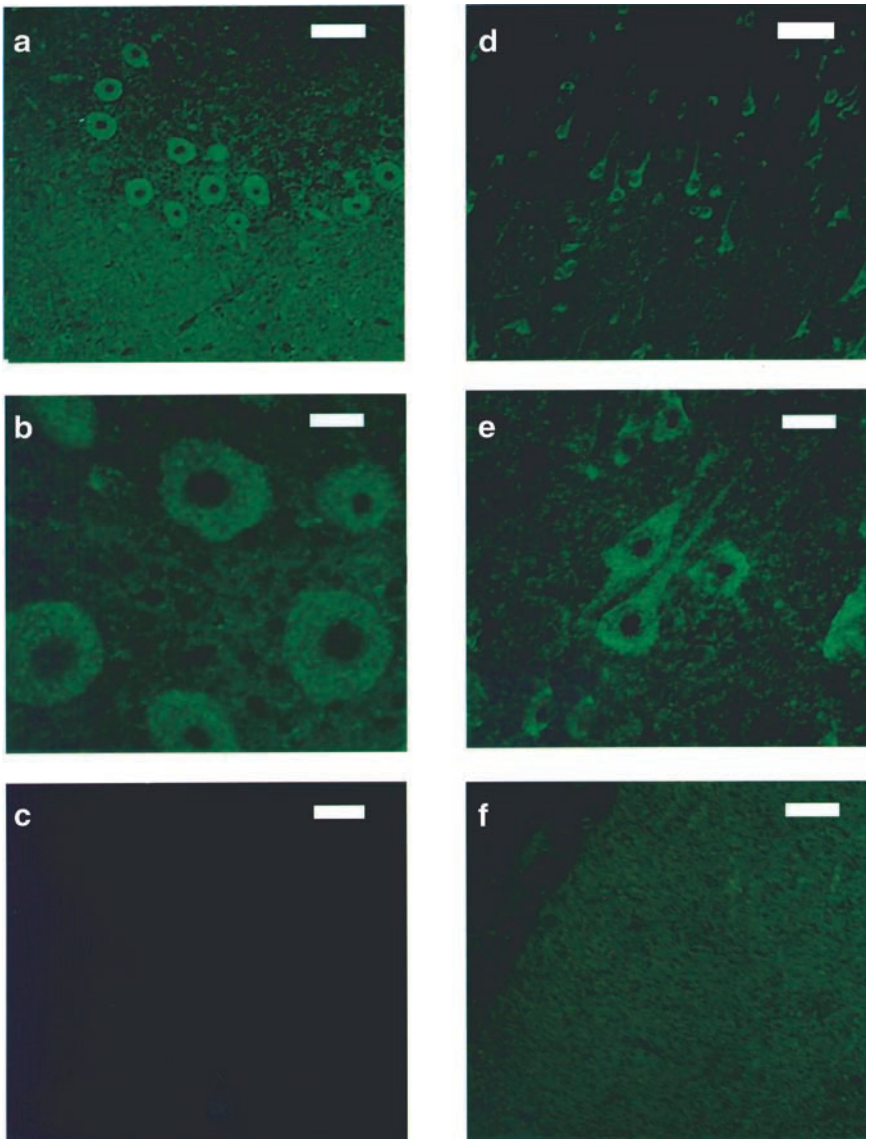

Figure 13 Localization of P4503A in human brain cortex and cerebellum by immunofluorescence analyses. (a) Localization of P4503A in the Purkinje cell is depicted; Bar = 100 μm. (b) Higher magnification of the Purkinje neurons; Bar = 25 μm. (c) Control section incubated with non-immune serum did not show immunostaining in the cerebellum; Bar = 100 μm. (d) Immunolabelling of neurons in frontal cortex was seen indicating the presence of P4503A; Bar = 100 μm. (e) Higher magnification of the immunostained cortical neurons depicting the fluorescence in the apical dendrites; Bar = 25 μm. (f) No staining was seen in cerebral cortex section incubated with non-immune serum; Bar = 100 μm.

tetrazolium and 5-bromo 4-chloro 3-indolyl phosphate as chromogens.

Northern Blotting and Fluorescence *in-situ* Hybridization

cDNA to rat liver CYP3A1 was obtained as a gift from Dr Avadhani, USA, was used for the preparation of riboprobes. The total RNA was extracted from rat brain (cortex) and liver and human brain cortex as described by Chomezynski.[24] Total RNA was separated electrophoretically and transferred onto positively charged nylon membrane by capillary transfer,[25] UV cross linked and hybridized with digoxigenin labeled antisense riboprobe prepared using SP6 polymerase to CYP3A1. The sense cRNA probe was synthesized using T7 polymerase. The membrane was hybridized overnight with digoxigenin labeled sense/antisense riboprobe at 47°C, washed, incubated with antibody to digoxigenin Fab fragments conjugated with alkaline phosphatase. The bands were visualized using chromogenic substrate for alkaline phosphatase.

Fluorescence *in situ* Hybridization

Male Wistar rats were anesthetized and perfused transcardially with normal saline followed by buffered paraformal-

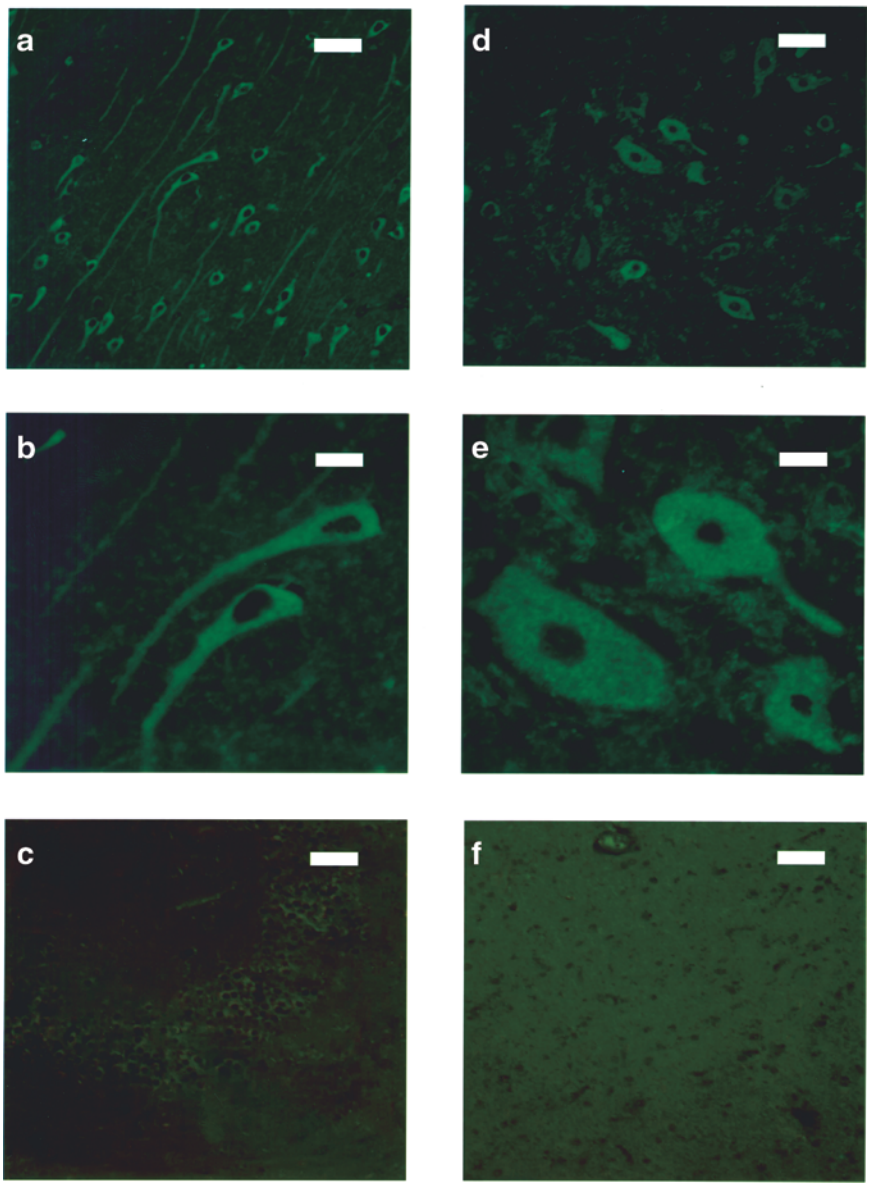

Figure 14 Immunofluorescence localization of P4503A in human brain hippocampus and midbrain using antiserum to P4503A4. (a) Immunostaining of CA1 pyramidal neurons indicating the presence of P4503A; Bar = 100 μm. (b) Higher magnification of the immunostained neurons showing the staining of the axons; Bar = 25 μm. (c) Similar section treated with non-immune serum did not show immunostaining of the CA1 neurons; Bar = 100 μm. (d) Immunostaining of reticular neurons in the midbrain indicating the presence of P4503A was observed; Bar = 100 μm. (e) Higher magnification of the reticular neurons; Bar = 25 μm. (f) A similar section of midbrain treated with non-immune serum did not show immunostaining; Bar = 100 μm.

dehyde (4%, w/v; 200 ml per rat) prior to the removal of brain. Different regions from human brain (cortex, cerebellum, hippocampus) were dissected out and fixed in buffered paraformaldehyde. The tissue was processed for paraffin embedding and serial sections (8–10 μm thick) were cut under RNase-free conditions. Sections were dewaxed, hydrated in graded ethanol, acetylated and treated with proteinase-K. The sections were then rinsed in phosphate–buffered saline and dehydrated using graded ethanol. Digoxigenin labeled sense (for control sections) and antisense cRNA probes were synthesized from CYP3A1 cDNA using T7 and SP6 RNA polymerases, respectively. Sections were hybridized overnight at 42°C with sense or antisense probes. After hybridization the sections were washed, incubated with blocking reagent (0.5%, w/v, NEN Life Sciences, USA) and incubated with antibody to digoxigenin conjugated to horse radish peroxidase. After washing, the sections were incubated with biotinylated tyramide followed by streptavidin fluorescein. Finally the sections were washed, dried and mounted prior to examination under the fluorescence

microscope.

Immunohistochemistry

Paraffin embedded sections were prepared from rat and human brain tissue as described above. Sections were dewaxed and transferred to phosphate buffered saline containing hydrogen peroxide (3%, v/v) to block the endogenous peroxidase reaction. The sections were pressure cooked in sodium citrate buffer (0.01 M, pH 6) for antigen retrieval. The sections were blocked with normal goat serum and incubated with antibody to hepatic P4503A4. Control sections were incubated with non-immune serum. The sections were washed, treated with biotinylated anti-rabbit IgG and incubated with VECTASTAIN–Elite ABC reagent. The color was developed using diaminobenzidine and hydrogen peroxide. The sections were washed, dried and mounted before examination under the microscope. For immunofluorescence studies, after incubation with the primary antiserum, the sections were incubated with anti-rabbit IgG labeled with peroxidase. After washing, the sections were incubated with biotinylated tyramide followed by streptavidin fluorescein. Finally the sections were washed, dried and mounted prior to examination under the fluorescence microscope.

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DUALITY OF INTEREST

None declared.

Abbreviations

cytochrome P450–P450; 4-hydroxy alprazolam—4-OHALP; α-hydroxy alprazolam—α-OHALP

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