Brain Parenchymal Metabolism of 5-Iodo-2′-Deoxyuridine and 5′-Ester Prodrugs

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In an attempt to generate derivatives of 5-iodo-2′-deoxyuridine (IDU) with enhanced blood–brain barrier (BBB) permeability, a series of 5′ ester prodrugs of IDU was synthesized and their metabolism studied in rat brain homogenate and its different subcellular fractions. The rate of hydrolysis was dependent on the steric and polar nature of the ester substituent. Ester hydrolyzing activities were associated primarily with the cytosolic fraction and were due mainly to the presence of cholinesterases as confirmed by inhibition experiments performed with different esterase inhibitors. The metabolism of IDU to 5-iodouracil (5-IU) by the cytosolic fraction, in the presence and absence of specific pyrimidine nucleoside phosphorylase inhibitors, also suggests that there are two specific enzyme systems catalyzing two different metabolic processes. IDU 5′-esters competitively inhibit the metabolism of IDU and the inhibitory effect depends on the affinity of a particular ester toward the enzyme and also on the rate by which the ester itself undergoes hydrolysis. In the absence of any 5′-ester, 95% IDU was metabolized within 6 hr. However, in the presence of an eightfold molar excess of butyryl-IDU, the hydrolysis of IDU was completely inhibited over a 6-hr time period.

KEY WORDS: 5-iodo-2′-deoxyuridine; 5′-ester prodrugs; metabolism; brain; cytosolic fraction; esterase activity; phosphorylase activity; competitive inhibition.

INTRODUCTION

Idoxuridine (IDU; 5-Iodo-2′-deoxyuridine), a thymidine analogue, is a topical antiviral agent indicated in the treatment of ocular herpes simplex infections (1,2). Its therapeutic effectiveness in the treatment of herpes simplex encephalitis, however, has been questionable (3,4). It is likely that the ineffectiveness arises from its polar and hydrogen-bonding interaction with water, which restricts transcellular transport across lipoidal brain capillary endothelium. The lack of a specific carrier at the blood–brain barrier (BBB) interface for thymidine analogues may also be responsible for inadequate transport across the BBB. Thus, it would be most desirable to enhance IDU transport to the brain in a sustained manner.

Enhanced drug transfer across the BBB has been attempted utilizing various novel strategies (5). One such strategy is the development of lipophilic prodrugs from highly water-soluble drugs by blocking hydrogen bond-forming sites through relatively lipophilic groups (6). If the brain has the enzymic capability of removing these added lipophilic groups, the original compound is regenerated after it has penetrated the BBB. One strategy in this regard is the coupling of water-soluble drugs to a pyridine nucleus (5). The coupling greatly enhanced amount of the derivatives in the brain because of increased lipophilicity, and once in the brain extracellular fluid, brain oxidative enzymes convert the pyridine base to a quaternary pyrimidine salt which entraps the drug in the brain cellular compartments (5).

A similar strategy has been adopted in this project to promote delivery of a polar nucleoside, IDU, to brain using lipophilic 5′-ester prodrugs. These compounds were designed to provide suitable prodrug forms that would possess increased lipophilicity and hence increased potential for BBB transport. The compounds are expected to undergo cleavage to the parent compound within the brain parenchymal cells. Once the polar IDU is regenerated in the brain parenchyma, it is expected to be "locked in" the brain because it cannot readily diffuse out through the BBB into the systemic circulation. The availability of a greater amount of IDU in the brain may be obtained not only by enhanced BBB transport but by reduction in the activity of the catabolic enzymes responsible for the cleavage of IDU to 5-iodouracil (5-IU). Several attempts have been made in the past to achieve and maintain therapeutic concentrations of the drug, which include the use of increased amounts and frequency of administered IDU (7), the coadministration of amethopterin (7), 5-fluoro-2′-deoxyuridine (9), and 5-iodouridine (8), the use of an oil emulsion of IDU to provide a depot (8), and prior treatment with thymine ribonucleoside (10).

In an earlier report, we described the synthesis of a series of IDU-5′-ester prodrugs (11) and their physicochemical and protein binding properties relevant to BBB transport (12). The present study investigates the enzyme kinetics of IDU and its 5′-ester metabolism by brain cytosolic fractions and the mechanism of inhibition of IDU catabolism by 5′-esters.

MATERIALS AND METHODS

Materials

The parent drug 5-iodo-2′-deoxyuridine, 5-iodouracil, phenylmethylsufonyl fluoride (PMSF), diisopropyl flurophosphate (DFP), *p*-chloromercuriphenyl sulfonic acid (PCMSA), *p*-chloromercuribenzoic acid (PCMB), *p*-hydroxymercuribenzoic acid (PHMB), and 5′5′-dithio-bis(2-nitrobenzoic acid) (5,5′ DTNB) were obtained from Sigma Chemical Co., St. Louis, MO. The chemicals and solvents used were of reagent grade and were used as received. The ester prodrugs of IDU were synthesized by esterification of the 5′-hydroxyl group of IDU using a 1.1 molar excess of appropriate acid chloride in a pyridine–dimethyl formamide solvent (1:1). The details of the synthetic methodology are described in our previous report (10).

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Methods

Enzymatic Hydrolysis of Various 5'-Ester Substrates

Male Sprague–Dawley rats weighing 300–350 g were sacrificed by decapitation and the brains were immediately removed, weighed, and homogenized in 5 vol of 0.01 *M* Tris–HCl buffer (pH 7.4) containing 0.25 *M* sucrose and 1 m*M* EDTA. The homogenate was centrifuged at 10,000 *g* for 20 min and the resulting supernatant was further centrifuged at 105,000 *g* for 60 min (Beckman TL 100) to obtain the cytosolic fraction. The fractions were stored in small aliquots at −20°C until further studies. When required, the frozen samples were rapidly thawed prior to use, and once thawed, unused portions were never refrozen and not used.

Twenty-five microliters of a freshly prepared solution of IDU and its 5'-ester derivatives (15 m*M*) was added to 475 μl of cytosolic fraction, previously equilibrated at 37°C, resulting in an initial substrate concentration of 0.75 m*M*. An aliquot (25 μl) of the sample was withdrawn at different time points and the reaction was stopped by adding 15 μl of ice-cold methanol. Following thorough mixing, the solution was centrifuged at 8000 *g* for 10 min and the supernatant was analyzed by HPLC as reported previously (11). The percentage substrate remaining was calculated from the control experiment where buffer was used instead of cytosol.

Identification of the IDU Metabolite

Following enzymatic cleavage of IDU by the cytosolic fraction of the rat brain, the mixture was centrifuged and the supernatant was fractionated by reversed-phase HPLC as described earlier (11). The metabolic product (retention time, 6.14 min.) was collected, lyophilized, and analyzed by HPLC and TLC [using chloroform–isopropyl alcohol (5:1) as the developing solvent] and also by mass spectrometry. Both electron impact (EI) and isobutane chemical ionization (CI) detections were used (Finnegan 4000, Finnegan MAT, San Jose, CA). The EI spectrum of the metabolite produced only low mass fragments. However, CI generated an MH^+ ion. This technique enabled us to control the tendency of the CI-produced MH^+ ion to fragment and also, generated a large MH^+ peak for easy identification.

Enzymatic Hydrolysis of IDU and 5'-Ester Prodrugs in the Presence of Inhibitors

In order to identify the enzyme classes involved in the bioconversion of both IDU-5'-ester derivatives and parent IDU in the brain tissue, *in vitro* metabolism studies were carried out in both the presence and the absence of specific inhibitors for esterases (13,14) and pyrimidine nucleoside phosphorylases (15–17). For esterase inhibition studies, 5 μl of an inhibitor solution (DFP or PMSF) was first incubated with 235 μl of the brain cytosolic fraction for 15 min. Following this period a second incubation was initiated by adding 10 μl of a 30 m*M* solution of either valeryl IDU or IDU to the cytosolic fraction, thus generating an initial substrate concentration of 1.2 m*M*. Similarly for pyrimidine nucleoside phosphorylase inhibition studies, 5 μl of 1.5 m*M* inhibitor solutions (PCMB or PHMB or PCMSA or 5,5'-DTNB) was first incubated with 42.5 μl of cytosolic fraction for 15 min. The second incubation was then initiated by adding 2.5 μl of a 15 m*M* solution of either butyryl IDU or IDU, thus generating an initial substrate concentration of 0.75 m*M*. In both experiments the mixtures were incubated for 60 min at 37°C and the reactions were arrested by adding 4 vol of ice-cold methanol. The mixtures were subsequently centrifuged at 8000 *g* for 10 min and the supernatants were analyzed for the remaining substrate concentration by HPLC. For control experiments, cytosol and inhibitors were replaced with the same volume of blank buffer solutions.

Effect of IDU Esters on the Metabolism of IDU

In order to examine the effect of IDU 5'-esters on the catabolism of IDU, an *in vitro* experiment was carried out in the presence of both IDU and butyryl IDU, with the ester-to-IDU molar ratio fixed at 8:1. To an aliquot (475 μl) of the cytosolic fraction was added 5 μl of either 100 m*M* DFP or 100 m*M* PMSF and 10 μl of 300 m*M* butyryl IDU. The reaction was initiated by adding 10 μml of 37.5 m*M* IDU and the mixture was incubated at 37°C. Twenty-five-microliter samples were withdrawn from the mixture at different postincubation times and the reaction was stopped by adding 125 μl of ice-cold methanol. The samples were analyzed for the remaining IDU following centrifugation at 8000 *g* for 10 min. The percentage IDU remaining was calculated from the control experiments where IDU was incubated with buffer alone.

RESULTS AND DISCUSSION

The metabolism of IDU was determined *in vitro* using homogenates of subcellular fractions of rat brain. The loss of IDU in the cytosolic fraction was found to follow first-order kinetics. More than 80% of the total IDU was metabolized by cytosolic fraction within 4 hr. This observation suggests that the enzyme(s) responsible for metabolism of IDU is located in the cytosolic fraction of the rat brain. The metabolic product of IDU has the same HPLC retention time (6.14 min) and R_f values (0.64) on TLC plates as an authentic sample of 5-iodouracil (5-IU). Mass spectrometric results showed an ion at *m/e* 239, consistent with the protonated molecular ion peak. Mass spectral data confirmed the formation of 5-IU (*m/e* 239) from the catabolism of IDU. Therefore in the rat brain IDU is cleaved at the C–N bond between uracil and the deoxyribose moiety, similar to the pathway suggested by Prusoff *et al.* (7) regarding metabolism of IDU by rat liver. This result, however, is not in agreement with the findings of Weinstein and Chang (18), who suggested that IDU is rapidly deaminated by deaminases present in the rat brain and liver.

In order to address the question of whether or not the 5'-esters of IDU could undergo the necessary cleavage to release the parent compound, the hydrolytic cleavage of IDU esters was examined *in vitro* by the subcellular frac-

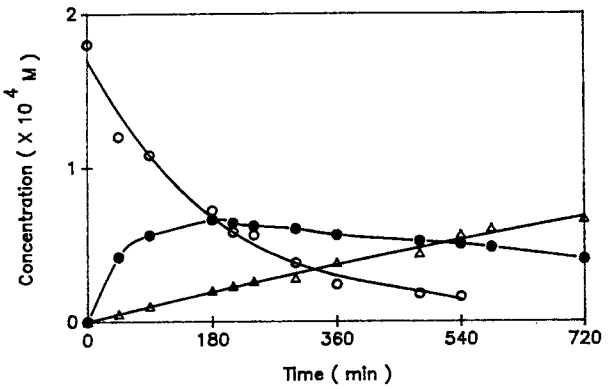

Fig. 1. Representative plots depicting the change in concentration of butyryl IDU (○——○), IDU (●——●), and 5′-IU (△——△) as a function of time in incubation media containing butyryl IDU in rat brain cytosol.

tions of rat brain. The hydrolysis of butyryl IDU to parent compound by the cytosolic fraction demonstrates the presence of esterases or esterase-like activity in the cytosolic fraction of brain (Fig. 1). Simultaneously with the disappearance of butyryl ester from the incubation mixture, the concentration of IDU increased to a maximum level in about 3 hr and then declined steadily. The level of 5-IU, on the other hand, increased continuously throughout the course of the experiment, suggestive of a precursor–product relationship of ester to parent compound to metabolite. No significant chemical hydrolysis was observed during the experimental time period, as the substrate concentration did not change in the control experiments where cytosol fraction had been replaced with an equal volume of buffer solution.

To identify the enzyme classes involved in these biotransformation processes, *in vitro* hydrolysis studies were conducted in the absence and presence of two different esterase inhibitors. As depicted in Fig. 2, in the absence of any inhibitors complete hydrolysis of butyryl IDU was observed within 180 min, while the addition of esterase inhibitors to the incubation medium either reduced the rate or completely inhibited the hydrolytic reaction. No 5-iodouracil was formed in the presence of the esterase inhibitors. The addition of 0.1 m*M* DFP resulted in complete inhibition of hydrolysis, while PMSF decreased the hydrolytic rate in a concentration-dependent manner. Thus the results presented here strongly suggest that the hydrolysis of the IDU 5′-esters is due mainly to the presence of cholinesterase activity in the cytosolic fraction of the brain parenchyma.

Figure 3 illustrates the hydrolytic conversions of various 5′-IDU esters in brain cytoplasmic fraction and the results are summarized in Table 1. For aliphatic esters, the maximum hydrolysis rate was obtained with the valeryl ester, followed by butyryl, propionyl, isobutyryl and pivaloyl derivatives in descending order. The differences in the rates of hydrolysis might be attributed to the polar and steric effects of the substituent. The relationship between increased linear chain length of the substrate and enhanced enzymatic hydrolysis might be attributed to the possible enhanced interaction between the substrate and a hydrophobic pocket at the active center of ester hydrolases, a phenomenon described for esterases responsible for the hydrolysis of ester prodrugs (19,20). However, an increase in the steric bulk of the 5′-acyl substituent tends to decrease the hydrolysis rate, i.e., pivaloyl < isobutyryl < *n*-butyryl. An increase in branching of the side-chain alkyl group appears to inhibit binding of the substrate to the active center of the enzyme. For aromatic esters, benzoyl ester appeared to be much more stable than the *p*-nitrobenzoyl ester. This could be explained by the possible electrostatic interaction between the ester and the cationic imadazolyl active site of the enzyme since the nitro group has strong electron withdrawing properties. The relative hydrolytic stability of anisoyl IDU also suggests the possible role of an electron donating anisoyl group in the electrostatic interaction between the ester molecule and the enzyme.

The esterase inhibitors had no effect on IDU metabolism (Fig. 4). Moreover, the effects of four inhibitors of phosphorylase-mediated metabolism of IDU demonstrate that the enzyme responsible for IDU metabolism is different from esterases. The pyrimidine nucleoside phosphorylase inhibi-

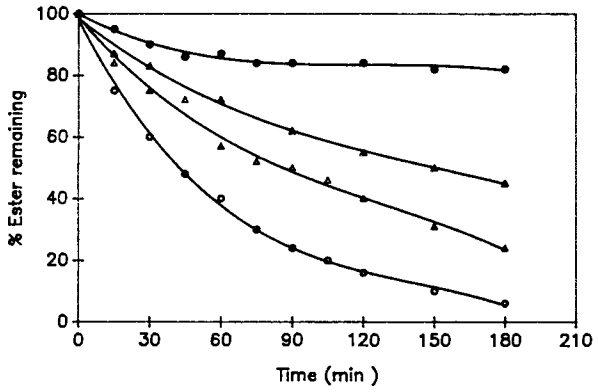

Fig. 2. Representative plots illustrating hydrolysis of the butyryl IDU as a function of time in brain cytosol in the presence and absence of the esterase inhibitors. (○——○) No inhibitor; (△——△) 0.1 m*M* PMSF (phenylmethylsulfonyl fluoride); (▲——▲) 1 m*M* PMSF; (●——●) 0.1 m*M* DFP (diisopropylfluorophosphate)

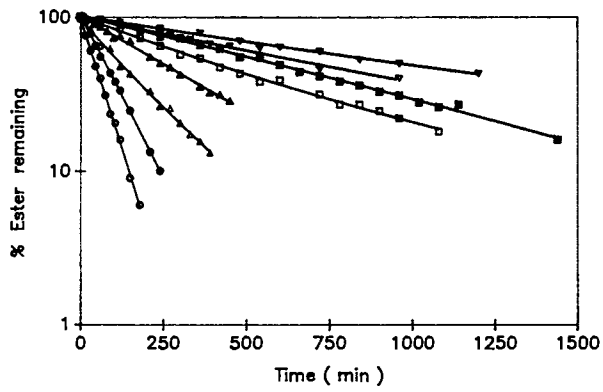

Fig. 3. Representative semilogarithmic plots indicating hydrolysis of various IDU esters in rat brain cytosol. (○——○) Valeryl IDU; (●——●) butyryl IDU; (△——△) propionyl IDU; (▲——▲) isobutyryl IDU; (□——□) *p*-nitrobenzoyl IDU; (■——■) pivaloyl IDU; (▽——▽) benzoyl IDU; (▼——▼) amisoyl IDU.

Table I. The Observed Hydrolysis Rate Constants for IDU 5'-Ester-to-IDU (k_1) and IDU-to-IU Conversion (k_2)[a]

Compound	Ester[b]		IDU[c]		
	$k_1 \times 10^3$ (min^{-1})	$t_{1/2}$ (min)	$k_2 \times 10^3$ (min^{-1})	$t_{1/2}$ (min)	$K_i \times 10^4$ (M)[d]
IDU	—	—	5.5	126	—
Propionyl IDU	5.2 ± 0.39	133.26	3.4	200	30.25
Butyryl IDU	9.7 ± 0.85	71.44	2.6	266	9.38
Isobutyryl IDU	2.8 ± 0.19	247.5	1.8	374	8.54
Valeryl IDU	16.2 ± 2.21	42.77	3.0	2315	
Pivaloyl IDU	1.2 ± 0.03	550	0.7	911	3.95
Benzoyl IDU	1.0 ± 0.02	693	0.5	1307	3.20
p-NO_2 benzoyl IDU	1.5 ± 0.06	462	0.9	753	14.28
Anisoyl IDU	0.67 ± 0.11	1034	0.29	2389	3.67

[a] The inhibitory efficiency of IDU-5'-ester on IDU-to-IU conversion in rat brain cytosol is represented by K_i values.
[b] Mean ± SD (n = 4).
[c] Calculated as the mean from the log ($A_0 - c$) vs t.
[d] Calculated from plots shown in Fig. 6.

tors were found to inhibit completely or partially the metabolism of IDU without affecting the ester hydrolysis (Fig. 5). This observation suggests that there are two enzymes involved in the cytosolic fraction of rat brain, one of which is responsible for the hydrolysis of IDU ester and the other for metabolism of IDU to 5-iodouracil (5-IU).

The esters appear to act as competitive inhibitors of the IDU metabolism in the brain (Fig. 6). The apparent K_m values for IDU metabolism increased in the presence of various 5'-esters. The degree of inhibition caused by IDU esters on the metabolism of IDU depends on the affinity of the esters toward the enzyme and also the rate by which the esters themselves undergo hydrolysis by esterases (k_1). The apparent first-order rate constant (k_2) for the catabolism of IDU (produced from the 5'-esters) to 5-IU can be computed by plotting the logarithm of the difference between the initial ester concentration and the concentration of the catabolic product (5-IU) formed at various time points. The rate constants for these processes are listed in Table I. The K_i values for different esters are also presented in Table I. The maximum inhibitory effect of benzoyl IDU on the metabolism of IDU could be explained by its high affinity for the enzyme (low K_i) and poor hydrolysis (low k_1) in the brain parenchyma. In comparison, propionyl IDU, which undergoes moderate hydrolysis and has the lowest affinity for the enzyme (high K_i), produces the least inhibitory effect on the metabolism of IDU. As can be seen (Fig. 7) all 5'-esters follow apparent first-order hydrolysis kinetics to generate IDU, which undergoes a slower rate of metabolism in the presence of intact 5'-ester.

As depicted in Fig. 6, the IDU 5'-esters compete with IDU to form a complex with the enzyme. Thus, the presence of ester decreases the rate of conversion of IDU to 5-IU since the ester first generates IDU before inhibiting IDU to 5-IU catabolism. Probably, the binding of the IDU-5'-ester with the enzyme changes the K_m of the enzyme for IDU. The enzyme catalyzing the breakdown of IDU has been identified as pyrimidine nucleoside phosphorylase (Fig. 5). It appears that first IDU-5'-ester is hydrolyzed by esterases to IDU, which is subsequently cleaved to IU by phosphorylase. The IDU esters competitively inhibit the IDU to IU catabolism. The degree of inhibition of IDU catabolism by its esters depends on both the concentration of the ester

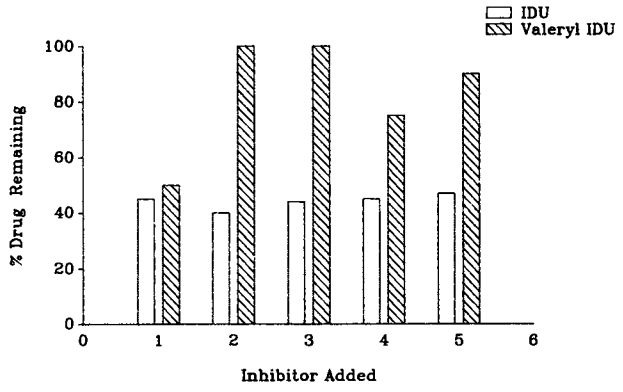

Fig. 4. Effect of esterase inhibitors on the hydrolyses of valeryl IDU and IDU in rat brain cytosol. (1) Tissue only; (2) 0.1 mM DFP; (3) 1 mM DFP; (4) 0.1 mM PMSF; (5) 1 mM PMSF.

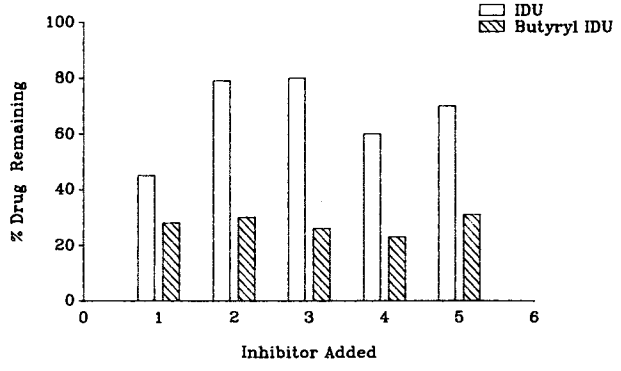

Fig. 5. Effect of pyrimidine nucleoside phosphorylase inhibitors on the hydrolyses of butyryl IDU and IDU in rat brain cytosol. (1) Tissue only; (2) p-chloromercuribenzoic acid; (3) 5,5'-DTNB; (4) p-chloromercuriphenyl sulfonic acid; (5) p-hydroxymercuribenzoic acid.

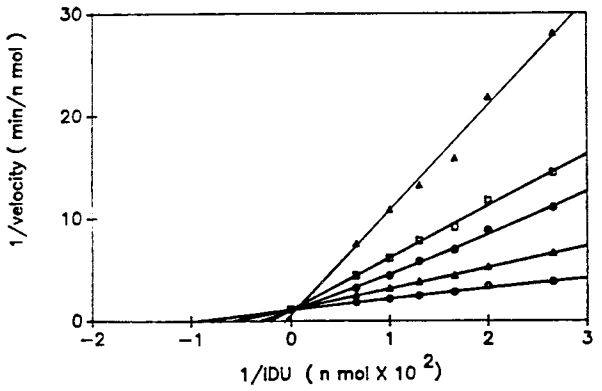

Fig. 6. Effect of increasing concentration of IDU on the rate of IDU metabolism in the presence and absence of different esters. The assays were carried out as described under Methods using varying concentrations (3.75×10^{-6}–15×10^{-6} *M*) of IDU and a fixed concentration (2.5×10^{-3} *M*) of IDU ester in a 100-μl reaction mixture. (○——○) IDU; (▲——▲) propionyl IDU; (●——●) butyryl IDU; (□——□) valeryl IDU; (△——△) benzoyl IDU.

remaining in the reaction mixture and the affinity of the esters for the phosphorylase.

The ineffectiveness of IDU in the treatment of brain viral infections can be attributed not only to its inability to cross the BBB but also to its rapid inactivation by pyrimidine nucleoside phosphorylase. Attempts to enhance its permeability through the BBB and also to prevent its rapid metabolism in the brain might improve the therapeutic efficacy of this antiviral agent in the treatment of viral encephalopathies. It is expected that IDU-5′-esters not only will enhance the ability of IDU to cross the BBB but also may enhance brain levels of the antiviral agent by inhibiting its metabolism in the brain parenchyma.

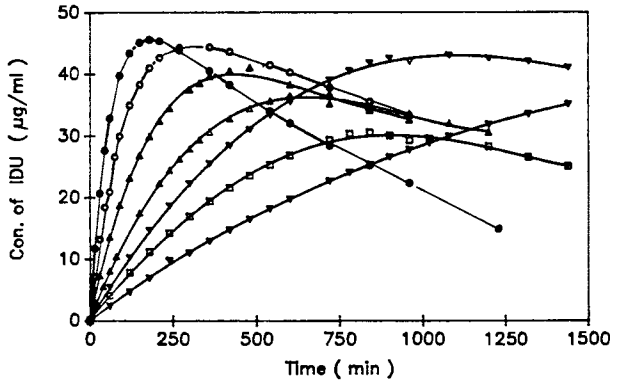

Fig. 7. The formation of IDU from different esters and its subsequent metabolism as a function of time. (●——●) Valeryl; (○——○) butyryl; (△——△) propionyl; (▲——▲) isobutyryl; (□——□) pivaloyl; (▽——▽) *p*-nitrobenzoyl; (▽——▽) anisoyl.

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