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Stereochemistry of pipecolic acid found in the urine and plasma of subjects with peroxisomal deficiencies

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Abstract: Recently it was found that normal adults excrete pipecolic acid primarily as the D-enantiomer even though it is present in the blood stream mainly as the L-enantiomer (i.e. >98% L). This study of pipecolic acid stereochemistry was extended to subjects with peroxisomal deficiencies since they are known to have high levels of pipecolic acid in their physiological fluids. Also, pipecolic acid stereochemistry was examined in young normal subjects since this group was not considered previously. It was found that the stereochemical composition of pipecolic acid in plasma was very similar for all subjects tested (i.e. >98% of the L-enantiomer). However, the stereochemical composition of excreted pipecolic varied considerably. Urine samples from subjects with the most severe peroxisomal deficiency, i.e. cerebralhepatorenyl (Zellweger) syndrome (CHRS) contained little D-pipecolic acid. In fact the enantiomeric ratios for pipecolic acid in the urine and plasma of these subjects were very similar. This was not the case for normal subjects. Levels of D-pipecolic acid in the urine of subjects with 'less severe' peroxisomal deficiencies tended to be somewhat higher but they did not approach the levels found in normal adults. Several possible reasons for these results are discussed.

Keywords: *D-pipecolic acid; L-pipecolic acid; Zellweger syndrome; chiral chromatography.*

Introduction

The level and distribution of amino acids in human physiological fluids has been studied for decades [1–12]. As the analytical methodologies for amino acids have improved so has the knowledge of the distribution and variation of amino acid levels in healthy human beings [3, 5, 11, 12]. Sometimes the level and disposition of certain amino acids can be of clinical and/or biochemical interest. Pipecolic acid is a case in point [13–19]. Pipecolic acid is not one of the 20 common amino acids found in proteins. It is a homologue of proline which is an imino acid [22]. Pipecolic acid is thought to be derived from the metabolism of lysine [20]. It has been reported to be a possible neurotransmitter [20], however its biological role is not well understood. Recent interest in pipecolic acid stems from the fact that elevated levels reportedly occur in subjects with cerebrohepatorenal (Zellweger) syndrome (CHRS). CHRS is a genetic disease that is invariably fatal. It is one of several related peroxisomal disorders (see Table 1).

Peroxisomes (formerly known as microbodies) are single membrane, subcellular organelles which are widely distributed in mammalian cells [21, 22]. They play a major role in a number of metabolic processes including the catabolism of long-chain fatty acids as well as other carboxylic, dicarboxylic and imino acids; the biosynthesis of bile acids and ether-phospholipids; and the oxidation of polyamines [13–22]. In 1973 Goldfischer *et al.* found that CHRS (the most severe peroxisomal disorder) was due to a lack of peroxisomes [23]. Subsequently a number of other related disorders were found in which there were decreased levels of peroxisomes or there were enzyme deficiencies in existing peroxisomes (Table 1).

Despite the extensive body of work on amino acids in physiological fluids, few studies have considered amino acid stereochemistry. It is generally assumed that amino acids in mammalian systems are of the L-configuration. Consequently even though the vast majority of amino acid measurements use nonstereoselective methods, it is assumed or sometimes tacitly implied that D-amino acids are not relevant.

Recent work has indicated that free D-amino acids are present in mammals in low to

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Table 1
Summary of some peroxisomal disorders[a]

I.	*Peroxisomes lacking or greatly diminished*
	A. Cerebrohepatorenal (Zellweger) syndrome[b]
	B. Neonatal adrenoleukodystrophy[c]
	C. Hyperpipecolic acidaemia[d]
	D. Infantile refsum disease[e]
II.	*Peroxisomes present but lack one function (single enzyme defect, SED)*
	A. Adrenoleukodystrophy (X-linked)[f]
	B. Thiolase deficiency[g]
	C. Acyl-CoA oxidase deficiency[h]
	D. Adult refsum disease[i]
	E. Acatalasaemia[j]
III.	*Peroxisomes present but lack several functions*
	A. Rhizomelic chrondrodysplasia punctata[k]
	B. Multiple β-oxidative enzyme deficiency[l]

[a]This summary was compiled from refs 19 and 24–27. As information on these diseases accumulate, this classification will continue to evolve and change as it has in the past.
[b]Usually fatal after several months, profound retardation.
[c]Usually fatal within ~1–2 years, profound retardation.
[d]Usually fatal within 1–3 years, severe retardation.
[e]Often fatal within ~10–15 years, severe retardation.
[f]Onset often occurs after ~5 years of normal development (although there is considerable variation). Often fatal ~2–5 years after onset.
[g]a.k.a. pseudo-Zellweger syndrome, only a few cases identified, usually fatal ~1–2 years if no treatment is possible.
[h]Often fatal within ~6 years, retardation.
[i]Often diagnosed by ~20th year. Major symptoms include retinitis pigmentosa and peripheral polyneuropathy. Often can be controlled by diet to prevent the accumulation of phytanic acid.
[j]Relatively benign disease where some subjects show a susceptibility for gangrenous oral lesions.
[k]Usual fatal in infancy, retardation, shortness of stature.
[l]Fatal, retardation.

moderate levels in virtually all physiological fluids [11, 12, 28]. Generally urine has the highest levels of D-amino acids although L-enantiomers still predominate [12]. However, pipecolic acid is somewhat unusual in that it appears to be excreted primarily as the D-enantiomer in healthy human, adult subjects [12]. Indeed it was not unusual to find that more than 90% of excreted pipecolic acid is of the D-configuration [12]. Conversely, pipecolic acid in plasma was primarily of the L-configuration (i.e. <2% D-pipecolic acid) [12]. It appeared that there was considerable variation in the stereochemistry of pipecolic acid when comparing plasma to urine. Consequently, it was decided to extend this investigation to subjects with known peroxisomal deficiencies as well as to healthy infants.

Materials and Methods

Apparatus

The chromatograph consisted of the following equipment from Shimadzu (Kyoto, Japan): three LC-6A pumps, a SCL-6B system controller, two CR601 chromatopac integrators, a SPD-6A UV–vis spectrophotometric detector, and a RF-535 fluorescence detector. Column switching and injection valves (models 7125 and 7010) were purchased from Rheodyne (Cotati, CA). The chromatographic columns used in this study were provided by Astec (Whippany, NJ) and included a C_{18} (3 μm, 100 × 4.6 mm), a C_8 (5 μm, 250 × 4.6 mm), and a β-cyclodextrin bonded phase (β-CD) (5 μm, 250 × 4.6 mm).

Materials

L- and D,L-pipecolic acid and 9-fluorenylmethylchloroformate (FMOC-Cl) were purchased from Sigma (St Louis, MO). Acetonitrile, methanol, and water were of OmniSolv grade and supplied by EM Science (Gibbstown, NJ). HPLC grade acetic acid and triethylamine were obtained from Fisher (Atlanta, GA). C_{18} (3 ml) disposable solid-phase extraction columns were purchased from Alltech (Deerfield, IL).

Urine and plasma samples from subjects with peroxisomal deficiencies were provided by Dr Ann B. Moser of the Neurogenetics Center at Kennedy Krieger Institute, Baltimore, Maryland. Normal infant plasma samples were provided by Dr Neal Grannemann of the Phelps County Regional Medical Center, Rolla, Missouri. Urine samples from healthy infants were provided by Dr Robert Huster of the Phelps County Regional Medical Center.

Procedure

Approximately 0.5–1.0 ml of plasma or urine sample was deproteinized by the addition of an equal volume of acetonitrile. The sample was centrifuged and transferred to another vial. Derivatization was performed by adding 0.2–0.5 ml of a 10 mM solution of FMOC-Cl reagent and 0.2 ml of 1% Na_2CO_3. After 20–30 min, the sample was acidified with 50 μl of acetic acid. The derivatized fluid was passed through a C_{18} solid-phase extraction cartridge. The adsorbed sample was washed with water to remove salts and then with acetonitrile. About 10–25 μl of the extract was injected into the achiral column. A UV wavelength of 266 nm was used to monitor the effluent. A typical achiral (C_8) separation of FMOC–pipecolic acid in a urine sample is shown in Fig. 1.

Normal samples were analysed according to the double column switching method described previously [12]. Samples from subjects with peroxisomal deficiencies contained higher levels of pipecolic acid and were injected into a C_{18} column and eluted with acetonitrile–water–acetic acid (44:56:0.2, v/v/v). The switching valve was turned for several seconds after the UV signal reached the maximum of the standard retention time. Therefore, a small portion of the eluting peak was introduced into the chiral column and eluted with acetonitrile–methanol–acetic acid–triethylamine (80:20:0.2:0.1, v/v/v/v). Figure 2 shows the enantiomeric separation of D- and L-pipecolic acid in plasma and urine of a healthy 5 month old child. All experiments were performed at room temperature and at 1 ml min^{-1} flow rate.

Quantitative measurement of the total pipecolic acid (i.e. D- plus L-enantiomers) in plasma and urine was done via a separate injection of the samples on both C_{18} and C_8 columns, respectively [12]. It is necessary to use fluorescence detection (λ_{ex} = 265 nm, λ_{em} = 315 nm) for accurate quantitation because much less sample could be injected. When using UV detection, a large amount of sample must be injected in order to detect pipecolic acid. This results in overloading and peak overlap (which makes quantitation inaccurate). Standard curves (i.e. fluorescence peak area vs concentrations) were made with pure FMOC–pipecolic acid (r = 0.997). The precision was determined at the 10 μmol l^{-1}

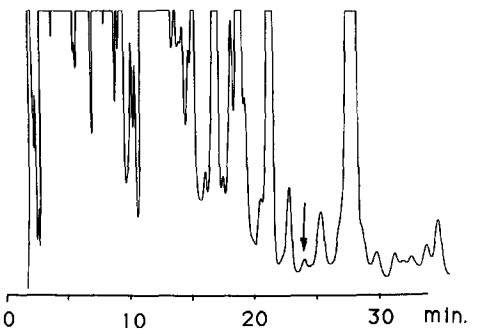

Figure 1
A chromatogram showing the achiral separation of FMOC-derivatized urine after solid-phase extraction. The arrow designates the peak containing pipecolic acid. Approximately 20 μl of sample was injected onto a C_8 column (250 mm × 4.6 mm i.d.) with 5 μ diameter packing. The mobile phase consisted of acetonitrile–water–acetic acid (440:560:2, v/v/v). The flow rate was 1.0 ml min^{-1} and UV detection was at 266 nm.

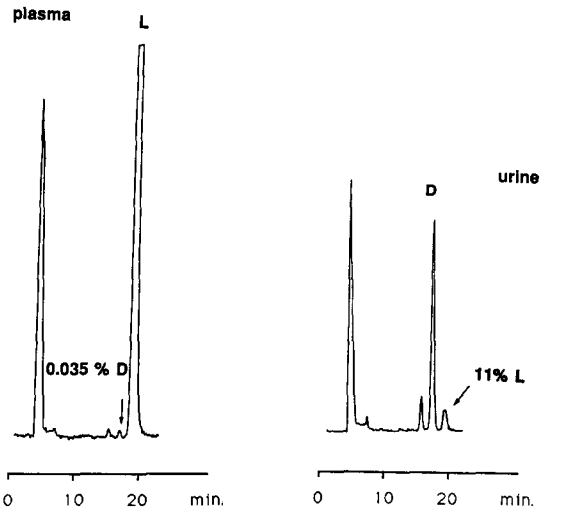

Figure 2
Two chromatograms showing relative amounts of D- and L-pipecolic acid in the plasma and urine of a normal 12 month old child. This separation of FMOC–pipecolic acid enantiomers was done on a Cyclobond I column (250 mm × 4.0 mm i.d.) with 5 μ diameter packing after 10 s switching from a C_{18} column (see Experimental). The mobile phase was acetonitrile–methanol–acetic acid–triethylamine (800:200:2:1, v/v/v/v). The flow rate was 1.0 ml min^{-1}. Fluorescence detection was used (λ_{ex} = 265 nm, λ_{em} = 315 nm).

level by measurement of six samples. The standard deviation of the peak area was 5.2%.

Results and Discussion

Urine and plasma samples from five subjects with cerebrohepatorenal (Zellweger) syndrome (CHRS) were analysed. In addition, samples from three subjects with neonatal adrenoleukodystrophy (NALD) and one sample from a subject with a single enzyme defect of peroxisomal fatty acid oxidation (SED-FAO) were tested. CHRS is a disorder in which the peroxisomes are missing. It is considered the most severe of the peroxisomal disorders [22–25]. NALD subjects also suffer multiple deficiencies or decreased levels of peroxisomal functions (Table 1).

Table 2 summarizes the pipecolic acid results for the plasma and urine samples from subjects with peroxisomal deficiencies. There were elevated levels of total pipecolic acid (i.e. D- plus L-enantiomers) in the urine and plasma of all subjects tested compared with normal adults [12]. This is consistent with a large body of prior work in this area, particularly for CHRS (Zellweger syndrome) [19–21]. As can be seen from the range of results, there was considerable sample to sample variation in the total pipecolic levels.

Stereochemical analysis of pipecolic acid produced very interesting results. The enantiomeric composition of pipecolic acid in the plasma of all subjects with peroxisomal deficiencies was very similar to that found for normal healthy adults (see Table 3 and ref. 12). In all cases the pipecolic acid in plasma was mainly of the L-configuration with only low levels of the D-enantiomers present (usually less than 2%). However, the stereochemical composition of excreted pipecolic acid (i.e. in urine) was found to differ considerably when comparing healthy adults to subjects with peroxisomal deficiencies. Normal adults tend to excrete relatively high proportions of D-pipecolic acid [12]. In fact, the majority of excreted pipecolic acid seemed to be of the D-configuration [12]. In contrast, the excreted pipecolic acid of subjects with CHRS was mainly of the L-configuration (Table 2).

Interestingly, the range and mean levels for excreted D-pipecolic acid of CHRS subjects was essentially the same as that found in plasma. This is almost never the case for normal adult subjects. In both human and rodent studies the urine generally contains higher relative levels of D-amino acids than does plasma (or other physiological fluids) [12, 28]. The reason for this is thought to be because L-amino acids are reabsorbed in the proximal tubules of the kidney in normal subjects [29, 30]. Hence the relative level of D-amino acids in urine is invariably higher than that found in plasma and most other physiological fluids [29, 30]. The relative level of D-pipecolic acid in the urine of normal human subjects was much higher than that found for most other D-amino acids (Table 3) [12]. Hence, it was surprising to find that CHRS urine samples contained much lower relative levels of D-pipecolic acid and that the relative amounts of D-pipecolic acid in the urine and blood were similar. One possible explanation for this is that CHRS subjects are not able to reabsorb L-pipecolic acid (or some other L-amino acids) as efficiently as normal subjects. Hence they would tend to excrete the same low relative level of D-pipecolic acid found in blood.

Another interesting facet of the data in Table 2 involves the relative level of D-pipecolic in the urine of neonatal leukodystrophy (NALD) subjects. In general, the NALD subjects seemed to excrete higher relative levels of D-pipecolic acid than the CHRS subjects (Table 2). However, these levels were nowhere near those found for normal adult subjects (Table 3) [12]. The reasons for this need to be investigated further. However, there are at least two possible mechanisms that could account for these results. One is that the efficiency with which a newborn's kidneys recycles L-amino acids improves somewhat as it matures (note that NALD subjects were usually >1 year old while CHRS subjects were usually a few months old) [31]. Alternately, relative D-pipecolic acid levels in the urine (relative to plasma) might somehow reflect the severity and/or nature of some peroxisomal deficiencies.

Previous studies on D-amino acid excretion by laboratory rodents indicated that a significant portion of these compounds are dietetic in origin [28]. Two factors were found that decreased the relative level of D-amino acids in urine. They were protein and amino acid depravation (e.g. fasting or starvation) and young age. Newborn laboratory rodents excreted lower levels of D-amino acids. This continued until they were weaned, after which

Table 2
Summary of pipecolic acid levels and enantiomeric composition in urine and plasma of subjects with peroxisomal deficiencies

	Plasma levels				Urine levels			
	Total pipecolic acid (μmol l^{-1})		% D-pipecolic acid		Total pipecolic acid (μmol l^{-1})		% D-pipecolic acid	
Disease	Range	Mean	Range	Mean	Range	Mean	Range	Mean
CHRS (Zellwegers syndrome)*	5–16	11.9	0.17–1.4	0.8	2–90	40	0.03–4	0.8
Neonatal adrenoleukodystrophy (NALD)†	3–24	13.6	0.2–0.3	0.2	2.5–18.3	8.3	1.8–30	12.7
Single enzyme defect‡	—	—	—	—	4.2	4.2	34	34

*Urine samples from five separate subjects were analysed three times each. Plasma samples from three subjects were analysed in the same manner (see Experimental).

†Urine samples from three separate subjects were analysed three times each. Plasma samples from three subjects were analysed in the same manner (see Experimental).

‡Only a single urine sample was available for testing. The single enzyme defect was in fatty acid oxidation.

Table 3
Summary of pipecolic acid levels and enantiomeric composition in urine and plasma of normal subjects

	Plasma levels				Urine levels			
	Total pipecolic acid (μmol l^{-1})		% D-pipecolic acid		Total pipecolic acid (μmol l^{-1})		% D-pipecolic acid	
Age of subject	Range	Mean	Range	Mean	Range	Mean	Range	Mean
20–94 years*	0.5–1.7	1.1	0.6–2.2	1.3	0.4–3.4	1.6	56–95	84
0.5–13 months† (unweaned)	0.6–1.1	0.9	0.02–1.6	0.8	1.0–1.8	1.4	3.0–3.5	3.2
1–24 months‡ (weaned)	0.8–1.3	1.1	0.5–3.0	1.5	0.4–2.1	1.2	41–95	64

*Data taken from ref. 12. Eight subjects tested.

†Urine samples from four separate subjects were analysed three times each. Plasma samples from three subjects were analysed in the same manner (see Experimental).

‡Urine samples from five separate subjects were analysed three times each. Plasma samples from three subjects were analysed in the same manner (see Experimental).

the relative concentration of D-amino acids rapidly approached that of adult levels. Thus, in one sense the newborn's low level of D-amino acids is related to diet as well, since its food source is derived from the mother's blood supply which is already partially cleared of D-amino acids via the kidney.

Because of the previous rodent study, it was decided to examine D- and L-pipecolic acid in normal human infants. The results are shown in Table 3. For comparison purposes, previously determined results for normal adults are included [12]. It appears that the plasma level for total pipecolic acid and D-pipecolic are very similar for all groups (i.e. adults, weaned infants and unweaned infants). Furthermore, the total pipecolic acid level of the urine was similar for all groups tested (Table 3). However the unweaned infants excreted far less D-pipecolic acid than either the adult subjects or their weaned counterparts. Despite this fact, the relative levels of D-pipecolic acid in unweaned infants' urine was still higher than that found for CHRS subjects (Table 2). The results in Table 2 suggest that the presence of D-pipecolic acid may be, to some extent, diet related. This is thought to be the case for protein amino acids [11, 12, 28]. If so, it may be possible to devise a controlled test of kidney function where oral administration of a quantity of D-pipecolic acid (or another D-amino acid) is followed by measurement and comparison of plasma and urine levels of the D- and L-enantiomers.

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