Pharmacokinetic Determinants of Embryotoxicity in Rats Associated with Organic Acids

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We have studied four organic acids of similar structure to further understand the basis of their developmental toxicity. Valproic acid (2-propyl pentanoic acid), ethylhexanoic acid, and octanoic acid are isomeric C8 organic acids but their teratologic potency varied widely. Valproic acid induced a moderate to severe teratologic outcome after a single oral administration of 6.25 mmoles/kg on day 12 of rat pregnancy. Twice as much ethylhexanoic acid (12.5 mmoles/kg) induced a less severe response. Octanoic acid was nonteratogenic even at the very high dose of 18.75 mmoles/kg. This latter result is undoubtedly due to poor intestinal absorption of octanoic acid, as the maternal plasma levels never reached half of those measured for valproic acid and ethylhexanoic acid. Moreover, only a tiny fraction of that in maternal plasma was actually transferred into the embryo. On the other hand, the peak concentration and duration of exposure to valproic acid and ethylhexanoic acid were very similar despite a more severe teratologic outcome following valproic acid, which indicated higher intrinsic activity of this latter agent. A fourth agent, methylhexanoic acid, was also studied and had no teratogenic effects when given at 14.1 mmoles/kg. Pharmacokinetic studies of this agent revealed higher peak concentrations in maternal plasma and embryo than valproic acid or ethylhexanoic acid, but the duration of exposure was shorter. We conclude that pharmacokinetic parameters can be important determinants of teratologic outcome and thereby help explain differing potencies of structurally similar chemicals. — Environ Health Perspect 102(Suppl 1):97-101 (1994)

Key words: valproic acid, ethylhexanoic acid, methylhexanoic acid, octanoic acid, transplacental distribution, litter effect

Introduction

Some years ago we indicated that most well-documented human teratogens are weak acids and speculated that, in part, this might be due to accumulation of acidic chemicals in the alkaline milieu of the early postimplantation embryo (1). In our present work, we have examined the teratogenic activity and transplacental distribution of three isomeric C8 organic acids, valproic acid (VPA, 2-propyl pentanoic acid), 2-ethyl hexanoic acid (EHXA), and octanoic acid (OA). The choice of these agents came from Ritter et al.’s study (2) showing that VPA was about twice as potent as EHXA in regard to developmental toxicity under identical dosage conditions in pregnant rats.

Malformations were mainly of the limb, tail, kidney, or cardiovascular system. The similar chemical structure and the similar malformation profile prompted these authors to speculate that these agents may work via the same mechanism.

A potential means by which the differing potencies of EHXA and VPA might arise is through some aspect of pharmacokinetics. It is our belief that both agents are teratogenic through a direct action of the parent compound on the embryo. Thus the transplacental distribution of each agent will be a major determinant of teratogenic potency.

Herein we examined the transplacental pharmacokinetics of VPA and EHXA after oral dosing on day 12 of rat gestation. Because the Wistar strain of rat used by Ritter et al. (2) could no longer be obtained, the present study used Sprague-Dawley rats, and a new teratology study was conducted to verify the different teratogenic potency of EHXA versus VPA in this rat stock. The straight chain C8 isomer, octanoic acid, was included in the study because other reports indicate a limited embryotoxicity associated with this agent in vivo (3) or in vitro (4).

As the study began, it was decided to add another agent, methylhexanoic acid (MHXA). This agent with one less carbon than the other organic acids was chosen because it was reported to possess very low embryotoxicity (4) but does have similar structure to the branched chain acids, VPA and EHXA. Our goal was to examine the association of embryotoxic response with transplacental pharmacokinetic distribution as a potential explanation for the differing teratologic potency of these chemically similar agents.

Materials and Methods

Studies were conducted with pregnant Sprague-Dawley rats purchased time-mated from Charles River (Portage, MI). The animals arrived at our laboratory on day 6 or 7 of pregnancy, allowing 5 to 6 days for acclimation prior to toxicant administration. Upon arrival, the females were housed in small groups in hanging wire cages in rooms maintained at a constant temperature (22 ± 1°C), relative humidity (50 ± 5%), under a controlled 12-hr light-dark cycle and fed Purina Rodent Laboratory Chow and water ad libitum.

EHXA and MHXA were purchased from Aldrich (Milwaukee, WI). VPA and OA were obtained from Sigma Chemical Company (St. Louis, MO). All four agents were administered undiluted by oral gavage on the morning of day 12 of rat gestation (day 0 = morning of finding vaginal plug).
Table 1. Embryotoxicity of C7 and C9 organic acids administered orally to Sprague-Dawley rats on day 12 of gestation.

| Agent | Dose, mmoles/kg | No. surv. females/no. treated | No. impl. sites | No. res. or dead (%) | No. surv. malf. (%) | Mean fetal wt., M/F |
|-------|-----------------|-------------------------------|----------------|---------------------|-------------------|-------------------|
| Control | 0               | 10/10                         | 108            | 6 (6)               | 1 (1)             | 3.90/3.71         |
| VPA   | 4.69            | 4/4                           | 56             | 10 (19)            | 5 (11)            | 3.39/3.03         |
| VPA   | 6.25            | 12/13                         | 115            | 33 (29)            | 50 (61)           | 2.65/2.66         |
| EHXA  | 12.5            | 9/9                           | 113            | 16 (14)            | 36 (37)           | 2.82/2.68         |
| EHXA  | 15.625          | 7/7                           | 78             | 47 (60)            | 31 (100)          | 2.06/1.93         |
| MHX A| 14.1            | 7/10                          | 81             | 2 (2)              | 0 (0)             | 3.29/3.46         |
| OA    | 18.75           | 11/12                         | 131            | 10 (9)             | 4 (3)             | 3.48/3.23         |

Teratology Studies

On day 20 of gestation, the rats were killed by chloroform overdose. The uterine horns were exposed through incision of the ventral abdominal wall and examined for placement of fetuses and counting of resorption sites. The fetuses were removed from the uterus, examined grossly for the occurrence of malformations, sexed, weighed, and then placed in Bouin's fluid (2/3) or in alcohol (1/3). Those in Bouin's fluid were subsequently examined by razor blade sectioning (5); those in alcohol were double stained for examination of cartilage and bone (6).

Transplacental Pharmacokinetics

At various intervals after dosing (0.25, 0.5, 1, 2, 4, 8, and 24 hr), pregnant rats were anesthetized with ether and the uterine horns were exposed through a ventral abdominal incision. Three to four embryos were dissected free of their surrounding membranes, dried of excess fluid with filter paper wedges, and placed in a preweighed Eppendorf tube and weighed on a five place electronic balance. Another three to four embryos were removed from the uterus with the membranes intact, and the exocoelomic fluid was removed by puncture of the membranes with a micropipette. This fluid was expelled into a preweighed Eppendorf tube for determination of toxicant concentration. The yolk sacs from these preparations were then collected and placed in a preweighed Eppendorf tube. Next, a sample of maternal skeletal muscle was taken from the anterior thigh and placed in a preweighed Eppendorf tube. Finally, a sample of maternal blood was withdrawn in a heparinized syringe from the abdominal aorta. This sample was centrifuged and the plasma removed to a preweighed Eppendorf tube. All samples were then frozen at −80°C until shipment on dry ice to Berlin, where the content of organic acid was measured by gas chromatography and mass spectrometry as described in detail by Fisher et al. (7). One or two remaining embryos were then examined under a dissecting microscope and the number of somites was counted as an indicator of actual embryonic age.

Results and Discussion

The developmental toxicity associated with administration of a C7 or C9 organic acid on day 12 of rat gestation is summarized in Table 1. MHXA and OA were essentially devoid of embryotoxic effects except for a slight reduction of fetal weight, most likely attributable to the severe maternal toxicity that accompanied the administration of these agents. This outcome was not unexpected because Nau et al. (3) showed that OA did not induce exencephaly when given to pregnant mice, and Brown et al. (4) showed that neither agent had any serious effect on rat embryos in culture at a concentration of 1 mM. VPA and EHXA were toxic to the 12-day rat embryo, causing increased death and malformation and a reduction of fetal weight. These results are also not surprising and agree reasonably well with an earlier report (2) from work done in Wistar rats. Both agents cause a similar spectrum of malformations when administered on day 12 of rat gestation and involve mainly the cardiovascular system and the appendicular skeleton. This similarity suggests that these two agents act by the same mechanism to divert embryonic development. In Table 2, which lists the most frequently occurring malformations there are some interesting differences that are dependent on the agent or strain of rat. With regard to cardiovascular malformations, VPA generally seemed to be more potent than EHXA and this was especially true with regard to alterations of the ductus arteriosus. In the present study utilizing

Table 2. Specific malformations induced by oral treatment with valproic acid or ethylxanoic acid on day 12 of rat gestation.

| Malformation                | Sprague-Dawley | Sprague-Dawley | Wistar | Wistar |
|-----------------------------|----------------|----------------|--------|--------|
|                            | VPA            | EHXA           | VPA    | EHXA   |
|                            | 6.25 mmoles/kg| 12.5 mmoles/kg| 6.25 mmoles/kg| 12.5 mmoles/kg|
| Externally examined         | 115            | 97             | 104    | 115    |
| Visceral exam               | 55             | 65             | 72     | 79     |
| Skeletal exam               | 27             | 32             | 32     | 36     |
| Cardiovascular              |                |                |        |        |
| Levo cardia                 | 17/55          | 3/65           | 15/72  | 5/79   |
| IV septal defect            | 2/55           | 1/65           | 4/72   | 2/79   |
| Truncus communis            | 1/155          | 1/65           | 7/72   | 0/79   |
| Double-outlet RV            | 15/55          | 0/65           | 6/72   | 4/79   |
| Ductus arteriosus           | 11/55          | 1/65           | 10/72  | 2/79   |
| Others                      | 35/55          | 2/65           | 5/72   | 8/79   |
| Axial skeleton              |                |                |        |        |
| Tail                        | 39/115         | 2/97           | 18/104 | 24/115 |
| Appendicular skeleton       |                |                |        |        |
| Entrodactyly, fl            | 21/115         | 17/97          | 1/104  | 31/115 |
| Bowed radii                 | 10/27          | 4/32           | 3/32   | 26/38  |
| Bowed ulnae                 | 4/27           | 0/32           | 1/32   | 11/38  |
| Polydactyly                 | 3/115          | 3/97           | 0/104  | 6/115  |
| Bowed fibulae               | 9/27           | 1/32           | 0/32   | 9/36   |
| Urogenital                   |                |                |        |        |
| Hydroleonephrosis           | 7/55           | 6/65           | 15/72  | 18/79  |

*Data from Ritter et al. (2). Includes short, missing, right-sided, dilated, or stenotic ductus arteriosus. includes free carotid agenesis, three dilated pulmonary artery, four right-side descending aorta, three pulmonary stenosis, two constricted aortic arch, two right-sided aortic arch; two double aortic arch, two ringed aortic arch, two dilated aortic arch, two misplaced carotid, eight valvula communis. Includes one carotid agenesis, one valvula communis. Includes two right-sided aortic arch, one carotid agenesis, one dilated pulmonary, one dilated aortic arch. Includes three ringed aorta, one right-sided aortic arch, one carotid agenesis, one pulmonary stenosis, one double aortic arch, one dilated aortic arch.
Sprague-Dawley rats, limb malformations were slightly more prevalent after VPA treatment. However, in the earlier study (2), EHXA clearly was more potent than VPA in this regard. Most often the limb malformations were bilaterally expressed and preferentially affected preaxial structures in the forelimb (bowed radii, ectrodactyly of digi 2), but postaxial hind limb structures (bowed fibula) were seen in Sprague-Dawley rats given VPA and in Wistar rats given EHXA. These findings have no credible explanations.

An interesting aspect of these results is the variation in frequency and severity of embryotoxic outcome between litters, especially in response to VPA (Table 3). Thus we had 7/12 litters in which all implantation sites were affected and one that had no embryotoxic effect. Furthermore, there was great disparity in the number of malformations in an individual fetus that was strongly correlated by litter. Thus, in two litters the malformed fetuses averaged approximately 13 malformations/individual (range = 8–22). In other litters, each individual had only one or two malformations. Both types of variation, frequency and severity of embryotoxic effect, were greatly reduced in litters exposed to EHXA. This same interlitter variability was evident in the previous study in Wistar rats (2).

This same trend of litter-specific effect is also evident in the fetal weight data. The standard deviation is less than 10% of the mean fetal weight for control animals but 3-fold higher for VPA-exposed fetuses (Table 4). Values for EHXA, MHX, and OA were intermediate between controls and VPA-exposed fetuses, but EHXA fetuses had the lowest variation of any of the treated embryos.

This great variation in developmental toxicity outcome between litters was also evident in the pharmacokinetic results in which the standard deviation from the mean of maternal plasma or embryo homogenate concentration was usually greater than 50%. We believe this variation of xenobiotic concentration between litters is real and based on three facts. First, when the same sample was rerun, the variation was within reliable limits. For example, six samples of embryos exposed to EHXA were reanalyzed two or three times and the standard deviation of the mean was less than 10% in all cases but one, where it was 13.3%. Similarly, in 12 samples of embryo homogenate exposed to MHXA variability was usually a little higher, approximately 20% of the mean. A second line of reasoning to uphold the idea of litter variability was the consistency of xenobiotic concentration in samples from the same animal. Thus, if maternal plasma contains a high concentration of xenobiotic, then other samples from the same litter (e.g., embryo homogenate, yolk sac, exocolomic fluid or maternal skeletal muscle) were likewise high; conversely, if maternal plasma was low, then these other samples were correspondingly low. The third factor indicating the validity of these variable results is related to the physical property of these agents as weak acids, which influences the extent of their distribution within a compartment based on pH. In Table 5 the free concentration of VPA in maternal plasma 4 hr after treatment shows great variability in the four mothers (29–530 mg/ml), yet the ratio of VPA in four compartments varying pH (8) is very consistent. This indicates that the variation among individual animals is real. Moreover, this variation of xenobiotic concentration from animal to animal is similar to the large interlitter variation of embryotoxic response and presumably is responsible for this litter effect. The basis of this pharmacokinetic difference (and presumably the dependent embryotoxic outcome) is unknown. The rats were not fasted overnight prior to early morning administration. Thus, varying amounts of gastric contents could have contributed to difference from rat to rat in the widely fluctuating maternal plasma concentrations.

The pharmacokinetic results provide additional insights into the teratologic potency associated with these structurally similar organic acids. Figure 1 depicts the concentration of each of the four acids in

### Table 3. Litter-specific developmental toxicity of VPA and EHXA given on day 12 of rat gestation.

| Litter no. | Total | Res. | Malf. | Malformations/ malf. surv. | Total | Res. | Malf. | Malformations/ malf. surv. |
|------------|-------|------|-------|---------------------------|-------|------|-------|---------------------------|
| 1          | 100   | 100  | 0     | —                         | 62    | 31   | 31    | 1.5 ± 1.0                 |
| 2          | 100   | 13   | 87    | 13.2 ± 3.1                | 36    | 14   | 21    | 3.7 ± 4.6                 |
| 3          | 80    | 20   | 60    | 6.3 ± 9.2                 | 50    | 43   | 7     | 1.0 ± 0                   |
| 4          | 8     | 0    | 0     | 93 ± 93                   | 93    | 93   | 0     | 3.0 ± 1.5                 |
| 5          | 100   | 70   | 29    | 7.0 ± 7.5                 | 56    | 25   | 33    | 2.3 ± 1.5                 |
| 6          | 100   | 75   | 25    | 3.3 ± 1.2                 | 46    | 0    | 46    | 1.7 ± 1.2                 |
| 7          | 30    | 0    | 30    | 1.0 ± 0                   | 67    | 33   | 33    | 2.0 ± 0                   |
| 8          | 100   | 100  | 0     | 2.2 ± 1.8                 | 7     | 0    | 7     | 1.0 ± 0                   |
| 9          | 100   | 67   | 33    | 8.5 ± 0.7                 | 13    | 0    | 13    | 1.0 ± 0                   |
| 10         | 43    | 7    | 36    | 1.0 ± 0                   | —     | —    | —     | —                         |
| 11         | 45    | 9    | 36    | 1.5 ± 0.6                 | —     | —    | —     | —                         |
| 12         | 100   | 30   | 70    | 12.9 ± 4.3                | —     | —    | —     | —                         |

*Percentage of implant sites affected by death or malformation.

### Table 4. Weight of day 20 rat fetuses exposed to an organic acid on day 12 of pregnancy.

| Litter no. | Male | Female | Male | Female | Male | Female | Male | Female | Male | Female |
|------------|------|--------|------|--------|------|--------|------|--------|------|--------|
| 1          | 3.6  | 3.4    | 1.5  | 1.4    | 2.5  | 2.2    | 2.4  | 2.3    | 3.8  | 3.7    |
| 2          | 4.2  | 3.9    | 3.0  | 2.8    | 2.6  | 2.3    | 3.4  | 3.9    | 3.7  | 3.7    |
| 3          | 4.0  | 3.7    | 3.2  | 3.0    | 2.6  | 2.3    | 3.7  | 3.7    | 2.5  | 2.2    |
| 4          | 4.0  | 3.9    | 2.5  | 2.4    | 2.7  | 2.4    | 3.9  | 3.9    | 3.5  | 3.3    |
| 5          | 4.0  | 3.8    | 2.5  | 2.2    | 2.6  | 2.5    | 3.9  | 3.9    | 3.5  | 3.2    |
| 6          | 3.9  | 3.7    | 3.2  | 3.1    | 2.8  | 2.8    | 3.9  | 3.9    | 3.3  | 3.2    |
| 7          | 3.5  | 3.4    | 3.3  | 3.1    | 2.4  | 2.4    | 3.7  | 3.7    | 3.8  | 3.7    |
| 8          | 4.3  | 4.1    | 2.3  | 1.9    | 3.0  | 2.8    | —    | —      | 4.1  | 3.9    |
| 9          | 4.5  | 4.2    | 3.4  | 3.1    | 3.0  | 3.1    | —    | —      | 2.2  | 2.0    |
| 10         | 3.9  | 3.8    | 3.2  | 3.1    | —    | —      | —    | —      | —    | —      |

| % variability | 7.5 | 7.2 | 21.3 | 23.2 | 12.9 | 16.3 | 17.3 | 18.5 | 19.1 | 20.7 |

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maternal plasma and embryo homogenate. The basis of low embryotoxic potential of OA becomes evident immediately. Very little of the administered dose reaches the maternal blood stream, and only a small fraction of that is transferred into the embryo. In contrast, MHXA, also of low embryotoxic potential, reaches a very high concentration in maternal plasma and embryo although it is eliminated more quickly than EHXA and VPA. Thus, the chemical structure of MHXA is sufficiently different from VPA and EHXA so that the cellular process disrupted by these teratogens is not susceptible to alteration by MHXA.

The levels of EHXA and VPA in both maternal plasma and embryo are roughly equivalent, despite giving twice as much EHXA to the maternal animal. This suggests that VPA and EHXA are equipotent within the embryo but that maternal pharmacokinetic factors lead to a more efficient uptake and transport of VPA. This conclusion is not borne out by study of VPA and EHXA in rat whole embryo culture (4) where EHXA was less potent than VPA. The embryonic concentration of either agent was not determined in this study so that pharmacokinetic factors such as protein binding could lead to different levels of teratogen in the embryo despite identical concentration in the culture medium (9).

**Table 5.** Ratio of VPA in embryonic, extraembryonic, and maternal compartments compared to the free concentration in maternal plasma 4 hr after intubation.

| Plasma-free | Embryo homogenate | Yolk sac | Exocoelomic fluid | Maternal muscle |
|-------------|------------------|----------|-------------------|-----------------|
| µg/ml       | plasma-free      | plasma-free | plasma-free | plasma-free |
| 530         | 1.06             | 0.83     | 1.25             | 0.38            |
| 29          | 1.10             | 1.10     | 1.45             | 0.38            |
| 296         | 1.09             | 1.05     | 1.56             | 0.48            |
| 120         | 0.98             | 0.87     | 1.16             | 0.37            |
| 244 ± 221   | 1.06 ± 0.05      | 0.96 ± 0.13 | 1.36 ± 0.18 | 0.40 ± 0.05 |
% variation  | 91               | 5        | 14               | 13              |

**Table 6.** Relative teratogenic sensitivity or resistance to four organic acids.

| Agent | Teratogenic Sensitivity | Explanation |
|-------|-------------------------|-------------|
| VPA   | ++++                    | High intrinsic activity |
| EHXA  | ++                      | Moderate intrinsic activity |
| MHXA  | +                       | Low intrinsic activity |
| OA    | +                       | Low embryonic exposure |

*Results extrapolated from Brown et al. (4) and Nau et al. (9).*

**Figure 1.** Disposition of four organic acids in maternal plasma (■) and embryo (○) on day 12 of rat pregnancy.

**Conclusion**

The work presented here indicates that pharmacokinetic characteristics of a chemical agent will influence the associated developmental toxicity; however, intrinsic activity of each agent is also an important determinant of teratologic outcome. Table 6 summarizes work done on the four organic acids studied herein, and indications of pharmacokinetic and intrinsic activity determinants of outcome are evident. MHXA distributes to the putative target site, the embryo, quite readily but is nonteratogenic at a concentration which VPA and EHXA induce severe developmental toxicity. OA had very low potential to induce developmental toxicity, which was clearly due to a low concentration in maternal plasma and very little placental transfer, presumably due to protein binding in maternal plasma (9). OA also possesses low intrinsic activity as shown in rodent whole embryo culture (4,9). It is surprisingly more active than VPA in some nonmammalian developmental systems (10).

Results from this work also indicate that pharmacokinetic parameters can help explain the varying teratologic response within members of the same species. Highly varying concentrations of VPA were correlated with a widely fluctuating teratologic response. An experimental design combining pharmacokinetic analysis and
teratologic examination in the same animal would permit a more direct answer to the basis of the so-called litter effect. This approach has been successful in the explanation of important pharmacokinetic determinants of salicylate teratogenicity (11) and warrants consideration for further studies with VPA and associated organic acids.

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