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Metabolism of N-phenyllinoleamide by rat liver

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ABSTRACT

N-Phenyllinoleamide (NPLA), the anilide of linoleic acid, has been associated with the epidemiology of toxic oil syndrome, but its contribution to the illness is still undetermined. Because it has been suggested that fatty acid anilides were absorbed via the hepatic portal vein, this study has been aimed at determining the hepatic metabolism of NPLA by rat liver. For this purpose, isolated liver was perfused with NPLA (0.1 m*M*) spiked with either aniline- or fatty acid-labelled NPLA. Gas chromatographic–mass spectrometric analysis of the peaks appearing in the radiochromatographic metabolic profiles shows that metabolism of NPLA in the liver results in formation of aniline and linoleic acid, both biologically active metabolites whose expected direct effects were not observed in patients suffering toxic oil syndrome.

INTRODUCTION

In 1981, a unique epidemic disease, characterized by pneumonitis, eosinophilia and marked loss of muscle mass function, made its appearance in Spain [1]. A number of epidemiological studies indicated the association between the illness and the consumption of an adulterated rape seed oil that had been denatured with aniline and illegally refined for the purpose of human consumption [2]. However, the nature of the substance(s) that persisted in the oil to render it so fiercely pathogenic is still unknown. The fatty acid anilides (FAA), including that of linoleic acid, which can be spontaneously obtained by the reaction of aniline with oil fatty acids during the refining process, transport and/or storage of these oils [3,4], are considered useful analytical markers of toxic oil syndrome (TOS)-related oils [2]. Nevertheless, the implication of FAA in the pathogenesis of TOS remains controversial. No correlation between cell toxicity and the anilide content of the implicated oils has been found to date. Also, synthetic anilides were found to be almost devoid of *in vitro* toxicity [5].

The fate of anilides in the organism is unknown, but factors directing their disposition, accumulation, biotransformation and elimination could be particularly significant for an understanding of the pathogenesis of the illness. It has been suggested that a first-step metabolic transformation in the liver could be possible [6], but no data are yet available on the identification of metabolites of FAA.

Taking into account that liver activity can either increase or decrease the toxicity of some xenobiotics, this study has been aimed to determine the hepatic metabolism of the linoleic acid anilide, or N-phenyllinoleamide (NPLA), using a rat liver perfusion system. For this purpose, we have applied gas chromatographic–mass spectrometric (GC–MS) techniques to the analysis of the metabolite peaks that appear in the radiochromatographic NPLA metabolic profiles.

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EXPERIMENTAL

Materials

Unlabelled NPLA, and NPLA labelled in the aniline (N-[ring-G-^{3}H]phenyllinoleamide, [^{3}H]NPLA; 154 mCi/mmol) or fatty acid (1-$^{14}C_{18:2}$, [^{14}C]NPLA; 62 mCi/mmol) moieties were prepared as previously described [7,8]. The [^{14}C]linoleic (^{14}C-L) acid (59 mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA).

N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), bovine serum albumin (fatty acid free and fraction V) and insulin were obtained from Sigma (St. Louis, MO, USA).

The commercial solutions of essential amino acids for minimum essential medium (50 ×) with L-glutamine, non-essential amino acids (100 ×) and BME vitamin solution (100 ×) were obtained from Gibco (Grand Island, NY, USA).

Other chemicals were obtained from Merck (Darmstadt, Germany).

Liver perfusion

Male Wistar rats weighing 180–200 g were anaesthetized by intraperitoneal administration of sodium pentobarbital (4 mg per 100 g body weight). The animals were cannulated according to the method of Meijer *et al.* [9], modified as follows. The portal vein was cannulated and perfusion was started at a flow-rate of 13 ml/min with medium A [136 m*M* NaCl, 54 m*M* KCl, 0.81 m*M* $MgSO_4$, 0.98 m*M* $MgCl_2$, 0.44 m*M* KH_2PO_4, 1.33 m*M* Na_2HPO_4, 1.3 m*M* $CaCl_2$, 5.5 m*M* D-glucose, 20 m*M* HEPES and 1% albumin (pH 7.4) gassed with O_2–CO_2 (95:5)]. The fluid was allowed to escape through a cut in the inferior cava vein at the level of the renal vein. The whole liver was carefully separated from non-hepatic tissues and placed in the perfusion chamber kept at 37°C. After 15 min of perfusion, remaining blood had been completely washed out of the liver. The perfusion medium was then changed to 40 ml of the medium B (same composition as medium A, with additional 0.3 n*M* insulin, 7% albumin and 2% amino acids and vitamins) supplemented with a mixture of 0.1 m*M* NPLA and spiked with either [^{3}H]NPLA (1 μCi) or [^{14}C]NPLA (1 μCi), which was recirculated for 1 h. Subsequently, the perfusion medium and the whole liver were stored at −80°C.

Extraction

NPLA metabolites in the acidified perfusion medium (pH 3) were extracted twice by addition of two volumes of ethyl acetate. The liver tissue was homogenized in 50 m*M* Tris buffer at 200 mg/ml, and the NPLA metabolites retained in the liver were extracted twice using water–methanol–chloroform (2:2:2, v/v/v). The ethyl acetate and chloroform extracts were evaporated to dryness under a helium stream, and the dry residue was resuspended in ethanol and stored at −80°C prior to high-performance liquid chromatographic (HPLC) analysis.

HPLC analysis

HPLC separations were performed on a reversed-phase 10 μm (30 cm × 0.4 cm I.D.) Spherisorb ODS-2 column (Tracer Analítica, Barcelona, Spain). The mobile phase was acetonitrile–water, to which acetic acid was added to pH 3.4; the flow-rate was 1.5 ml/min. A step-gradient programme consisting of 25 to 90% acetonitrile from 3 to 23 min and 90 to 100% in 5 min was used [10]. The radioactivity in the column effluent was continuously monitored using a flow detector coupled to a computer-regulated data processor (Ramona Ray Test, Isomess, Straubenhardt, Germany) or by radioactivity counting of sequential 30-s fractions in a liquid scintillation counter. Eluent fractions bearing the radiolabel tracer were individually collected for further analysis.

GC–MS analysis

The dried HPLC fractions corresponding to isolated peaks were analysed by GC–MS. Prior to their analysis, the metabolites were methylated with diazomethane and silylated with N,O-bistrimethylsilyltrifluoroacetamide (BSTFA), according to procedures previously described [11].

Mass spectral identifications were carried out under electron impact at 70 eV on a Finnigan-

TABLE I

PERCENTAGE OF TOTAL RADIOACTIVITY RECOVERED IN THE PERFUSED MEDIUM AND LIVER TISSUE

Perfusion was carried out with 0.1 m*M* NPLA containing either [^{3}H]NPLA or [^{4}C]NPLA as tracers. Results are the mean ± S.D. (n = 5).

Compound	Radioactivity in medium (%)	Radioactivity in liver (%)
[^{3}H]NPLA	61.2 ± 2.5	37.7 ± 1.8
[^{4}C]NPLA	7.0 ± 2.6	92.5 ± 3.2

Mat TSQ 700 mass spectrometer (Finnigan Mat, San José, CA, USA), at an acquisition rate of 1 scan/s. The spectra were scanned between 50 and 500 units. GC separations were performed on a 30 m × 0.25 mm I.D. (0.25 μm film thickness) fused-silica column coated with methyl silicone stationary phase. The GC temperature ramps used were 80°C for 0.5 min, 9°C/min to 200°C and then 4°C/min to 300°C.

RESULTS

The radioactivity distribution in the medium and in liver, after liver perfusion with either [^{3}H]NPLA or [^{14}C]NPLA, is summarized in Table I. The results clearly point to an active NPLA metabolization by the liver. According to these data, NPLA metabolites containing only the fatty acid moiety are mostly retained by the liver, whereas metabolites with the more polar N-phenylamide moiety are mostly released to the perfusion medium.

Fig. 1 shows the radiochromatographic profiles of perfused medium, after [^{3}H]NPLA perfusion (Fig. 1A), and liver tissue after [^{14}C]NPLA perfusion (Fig. 1B), compared with those from the non-perfused linoleic acid and NPLA standard tracer compounds. Comparison of ^{3}H and ^{14}C profiles indicates that liver metabolization of NPLA produces two metabolites (peaks 1 and 2 in Fig. 1) containing either the N-phenylamide or the fatty acid moiety, respectively. From the HPLC retention times of the standards (Fig. 1C), it could be established that peak 2 elutes at the position of linoleic acid, but its unequivocal identification was obtained by GC–MS assay. Under the GC–MS conditions indicated above, both el-

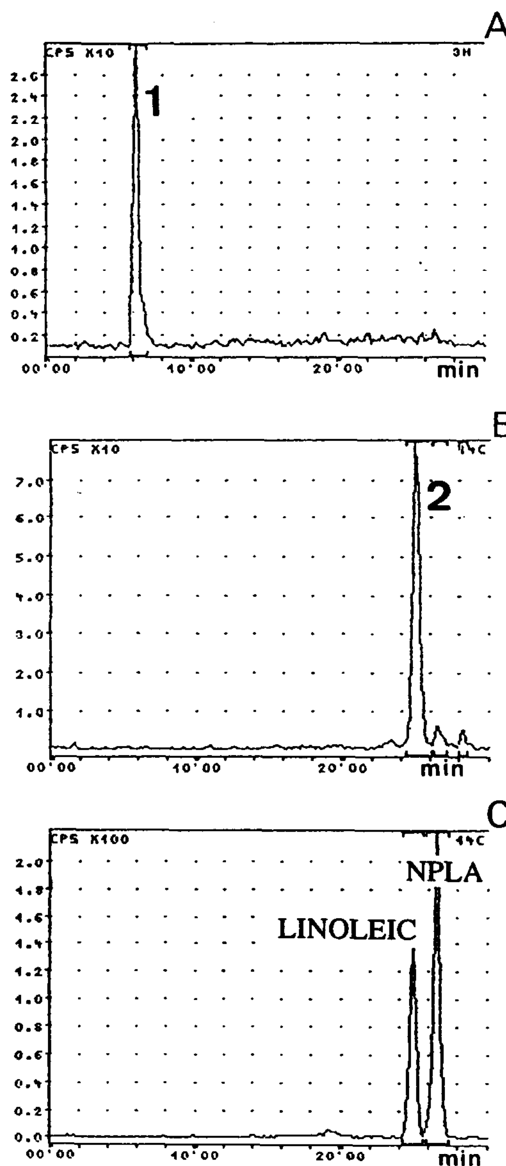

Fig. 1. Radiochromatographic profiles. (A) [^{3}H]NPLA metabolic profile of the perfusion medium from liver perfused with 0.1 m*M* NPLA and supplemented with 1 μCi of [^{3}H]NPLA; (B) [^{4}C]NPLA metabolic profile in liver tissue after liver perfusion with 0.1 m*M* NPLA supplemented with 1 μCi of [^{4}C]NPLA; (C) radioactive profile of standards of labelled linoleic acid and NPLA.

uate fractions (peaks 1 and 2) give clean single GC peaks at 6.25 and 17.30 min, respectively.

Fig. 2 shows the mass spectra obtained from an aniline standard (Fig. 2A) and the metabolite corresponding to peak 1 (Fig. 2B). Because spectra are identical, peak 1 is indeed aniline.

Fig. 3 shows the mass spectra of the methylated and silylated derivative (MeTMS) of a linoleic acid standard (Fig. 3A) and that of the metabolite corresponding to peak 2 (Fig. 3B). The spectra are coincident, so peak 2 is linoleic acid.

DISCUSSION

Data previously reported on the metabolism of the N-phenylamide of oleic acid suggested that it could be metabolized through hydrolysis of the amide bond [6,12]. Also, the liver has shown great affinity for oleylanilide, or related compounds, as reflected in a lower elimination rate [6] and the irreversible binding to liver macromolecules [13], but the molecular structures of oleylanilide-related metabolites have not been determined.

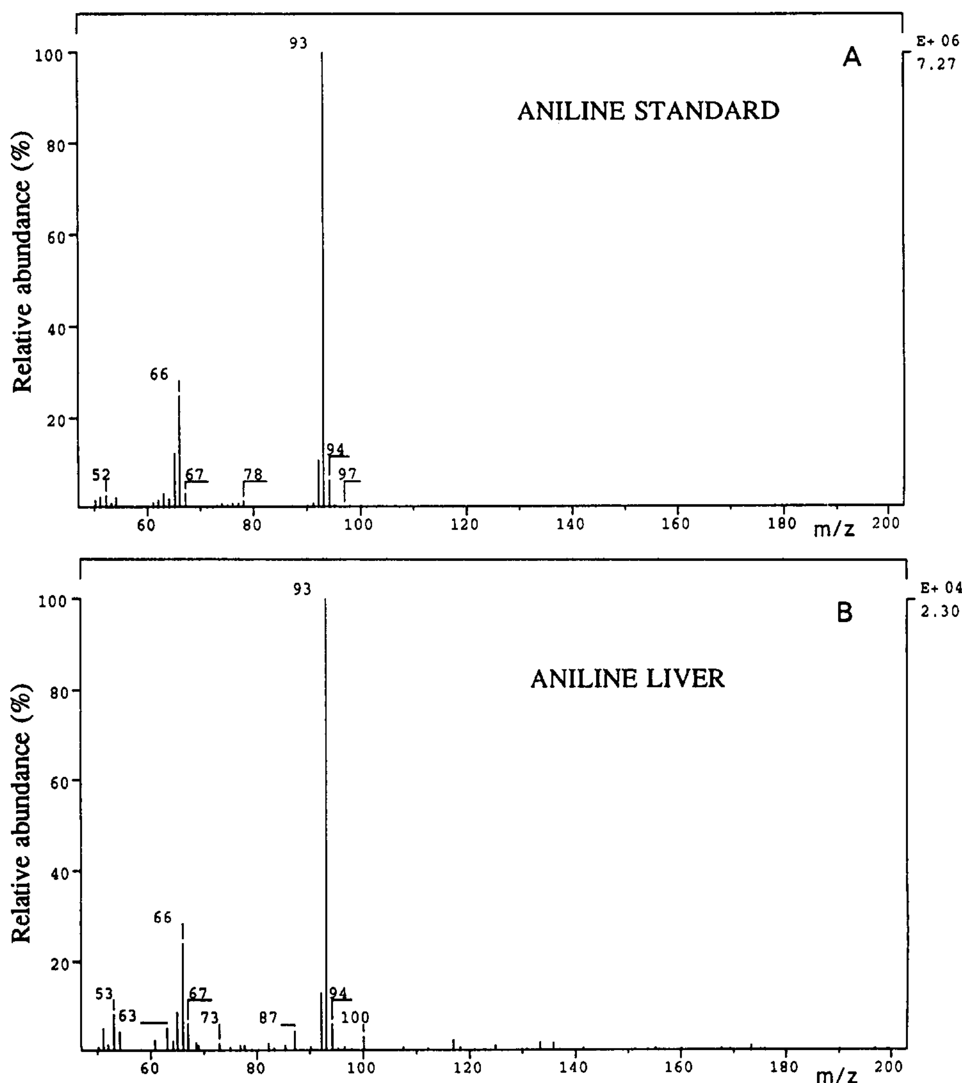

Fig. 2. Mass spectra of (A) standard aniline and (B) metabolically derived aniline in the perfusion medium (peak 1 in Fig. 1).

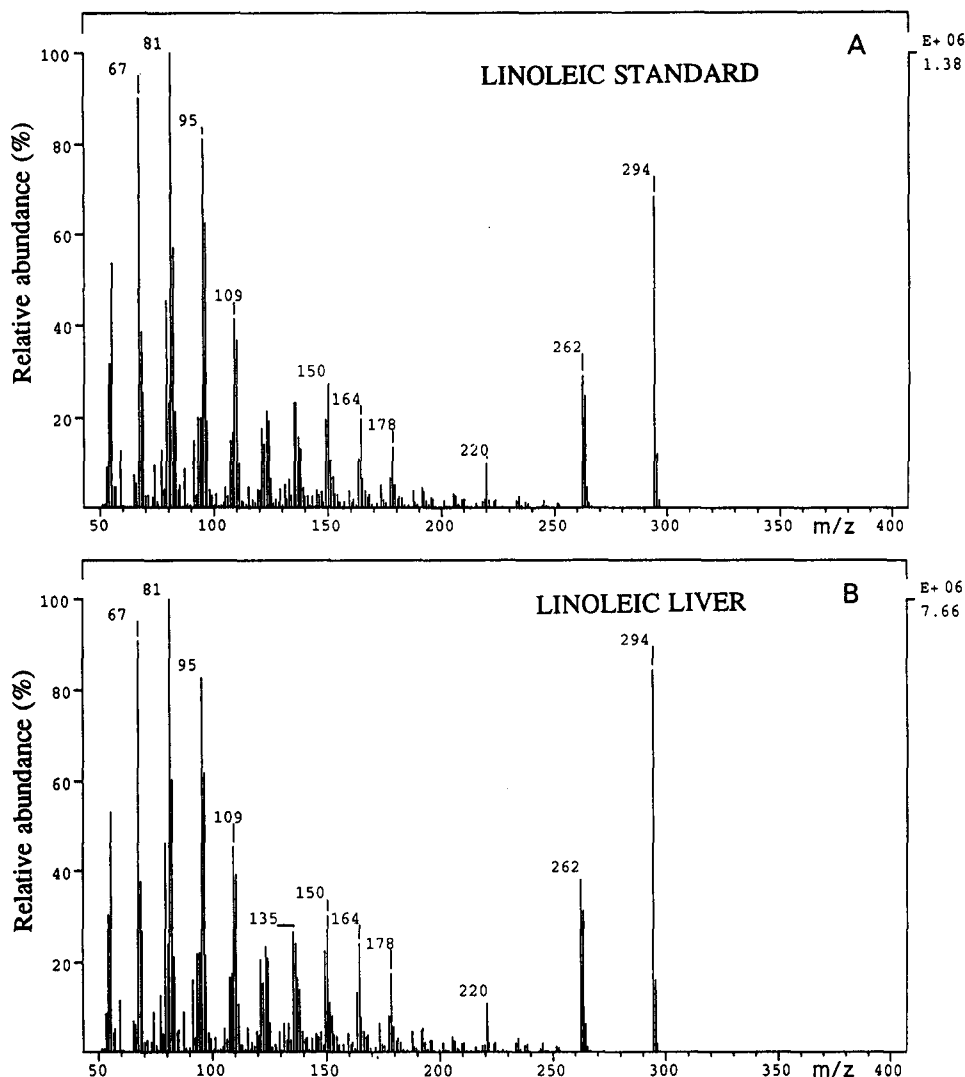

Fig. 3. Mass spectra of (A) the MeTMS derivative of standard linoleic acid and (B) metabolically derived linoleic acid in liver tissue (peak 2 in Fig. 1).

Our unequivocal identification of NPLA metabolites produced by rat liver suggest a hepatic amidase activity. This would be in line with other non-microsomal transformations reported for various substrates in liver, plasma and other tissues [14]. In this case, two metabolites, aniline and linoleic acid, are produced from the NPLA substrate. As indicated by the data in Table I, aniline could readily reach the systemic circulation while the linoleic acid is mainly retained in the liver. However, one must draw attention to the absence of methehemoglobinemia in TOS patients, which could be expected if free aniline had reached the blood compartment in sufficient concentration [15]. In any case, for methehemoglobinemia to occur, the aniline blood levels should be higher than 5% [16], an amount clearly much higher than could possibly result from liver metabolism of the low levels of ingested anilides. Reported anilide concentrations in TOS-related oils range from less than 0.5 ppm to 2000 ppm [5].

It has also been suggested that ingested FAA

could be distributed through lymphatic pathways directly to several tissues, including the lung, thus eluding a liver first-pass effect [6]. In this regard, it should be emphasized that, in the initial stages of the illness, some TOS patients showed lipid deposits on the vascular intima as well as Peyer's patches [17]. In tissues other than the liver, the anilides could be metabolized through oxidative pathways to derivatives with structures related to those resulting from the oxidative metabolism of linoleic acid [18], but differentiated by the phenyl ring and amide bond moieties present in the NPLA molecule. This would be in line with previously reported data on the oxidative metabolism of NPLA in human nasal polyps [19], in peritoneal mouse macrophages and polymorphonuclear lymphocytes [20].

In summary, our results show that metabolism of NPLA by rat liver results in biologically active metabolites whose expected direct effects were not observed in TOS patients. However, the role of NPLA and its metabolites in the illness cannot yet be dismissed because other metabolic routes may be possible and many factors could mediate the absorption and distribution of this xenobiotic in the organism.

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