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BIOTRANSFORMATION OF β-NORTESTOSTERONE BY CULTURED PORCINE HEPATOCYTES

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SUMMARY

The metabolism of β-nortestosterone by porcine hepatocytes was investigated. Initially β-nortestosterone was rapidly oxidized to norandrostenedione, which was further transformed into a number of more hydrophilic compounds. It is assumed that most of these compounds were glucuronides, considering the effect of β-glucuronidase treatment. The main product of enzymatic cleavage was investigated by gas chromatography–mass spectrometry but could not be identified until now.

INTRODUCTION

Primary cultures of hepatocytes might be a useful model for studying the biotransformation and toxic effects of foreign compounds [1]. An in vitro model with hepatocytes isolated from livers of food-producing animals might be even more advantageous, as biotransformation studies with large animals are expensive and require a number of special facilities. Previously we described the isolation and culturing of hepatocytes isolated from livers of sows [2]. In addition it was shown that these cells were able to metabolize two widely used antibacterial drugs, sulphadimidine and furazolidone, resulting in the formation of compounds previously detected in vivo.

Steroid hormones are a group of compounds with special interest in veterinary practice. Therefore, the biotransformation of β-nortestosterone (4-estren-17β-ol-3-one) (NOR) in monolayer cultures of porcine hepatocytes was investigated, using a radiolabelled compound in combination with a radioactivity detector coupled on-line to high-performance liquid chromatographic (HPLC) equipment. It is shown that NOR is rapidly oxidized, resulting in the formation of norandrostenedione (NA) and a number of conjugated metabolites.

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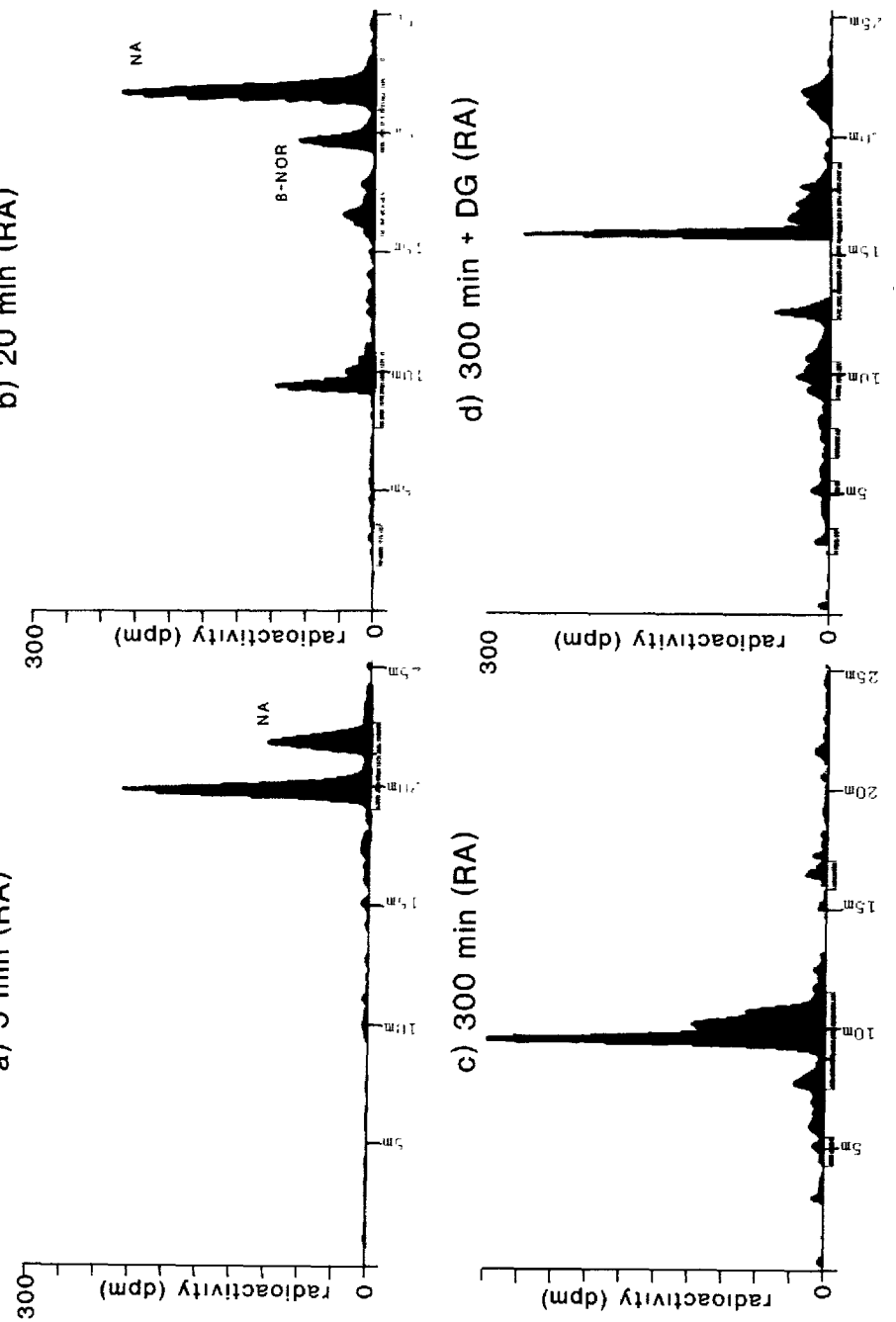

Fig. 1. HPLC of samples of incubation medium taken at (a) 5, (b) 20 and (c) 300 min after addition of β-nortestosterone to monolayer cultures of porcine hepatocytes. After 300 min the medium was aspirated, incubated with β-glucuronidase and analysed (d) (DG, deglucuronidation; RA, radioactivity detection).

EXPERIMENTAL

Hepatocyte isolation

Viable cultures of hepatocytes were isolated from a liver biopsy of a nine-month-old sow, pregnant for ten days, as described previously. In summary, the liver biopsy was perfused with an ethylene glycol bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid-containing buffer, followed by a buffer containing 0.05% collagenase (type I) (Sigma, St. Louis, MO, U.S.A.). Cells were collected, washed and cultured in Williams medium E (Flow Labs., Rickmansworth, U.K.) supplemented with 5% foetal calf serum (Flow Labs.), insulin (0.5 μg/ml) (Sigma) and penicillin–streptomycin (50 I.U., 50 μg/ml, respectively) (Flow Labs.) for 24–48 h. The cell density was about $3.5 \cdot 10^6$ per dish (60 mm) (Costar, Badhoevedorp, The Netherlands).

Incubation with β-nortestosterone

Prior to the addition of NOR, cells were washed and preincubated for 30 min in Krebs–Henseleit buffer containing 10 m*M* glucose and 20 m*M* HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid]. ^{3}H-Labelled NOR (703 GBq/mmol) (Amersham, Amersham, U.K.) was diluted in dimethyl sulphoxide with unlabelled NOR (Sigma) to a specific activity of 1.8 GBq/mmol. Incubation was started by the addition of 25 μl of this solution per dish (2.5 ml), resulting in a final concentration of 3.66 nmol/ml NOR (1 μg/ml). At various time intervals aliquots of 0.25 ml were taken and analysed by HPLC without further pretreatment. For deconjugation, samples were treated with β-glucuronidase (*Escherichia coli*) (Sigma) for 1 h at 37 °C.

HPLC analysis

Sampes were analysed on a PRP-1 column (250 mm × 4.1 mm I.D.) (Hamilton, Reno, NV, U.S.A.) using a 22-min linear gradient of 2–98% acetonitrile in water. Metabolites of NOR were detected using an on-line radioactivity monitor (LB506C; Berthold, Wildbad, F.R.G.).

Gas chromatographic–mass spectrometric (GC–MS) analysis

For identification of norandrostenedione [kindly supplied by the National Institute of Public Health and Environmental Hygiene (RIVM), Bilthoven, The Netherlands], monolayer cultures were incubated with unlabelled NOR at a final concentration of 36.6 nmol/ml. After 15 min the incubation medium was aspirated and analysed by HPLC. The fraction containing NA was collected, evaporated to dryness and derivatized by subsequent treatment with methoxylamine hydrochloride (MOX) (Chrompack, Middelburg, The Netherlands) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rockford, IL, U.S.A.) [3]. GC–MS analysis was performed as described previously [4].

RESULTS AND DISCUSSION

This study of the metabolism of NOR by primary cultures of porcine hepatocytes has shown that NOR was rapidly metabolized, resulting initially in the

formation of a compound coeluting with NA (Fig. 1). The identity was confirmed by GC–MS following derivatization of the two keto groups with MOX (Fig. 2). As a result of this treatment the molecular mass of 272 is increased by 58 (2×29) to a final mass of 330.

After prolonged incubation, NA was replaced with a number of more hydrophilic compounds, probably glucuronides (Figs. 1 and 3). This was confirmed by the fact that most of these compounds disappeared following treatment with β-glucuronidase. This resulted primarily in the formation of a compound eluting after 16 min (Fig. 1d). The glucuronide of this compound seems to be the major end-product of the biotransformation of NOR by primary cultures of hepatocytes isolated from the liver of a female pig. The identity of this compound is still under investigation.

The α-isomer (retention time 20.3 min) of NOR could not be detected in the incubation medium. In the absence of hepatocytes, prolonged incubation of NOR in Krebs–Henseleit buffer at 37°C for up to seven days gave no indication of chemical instability of this compound.

The results of this study clearly demonstrate the suitability of primary cultures

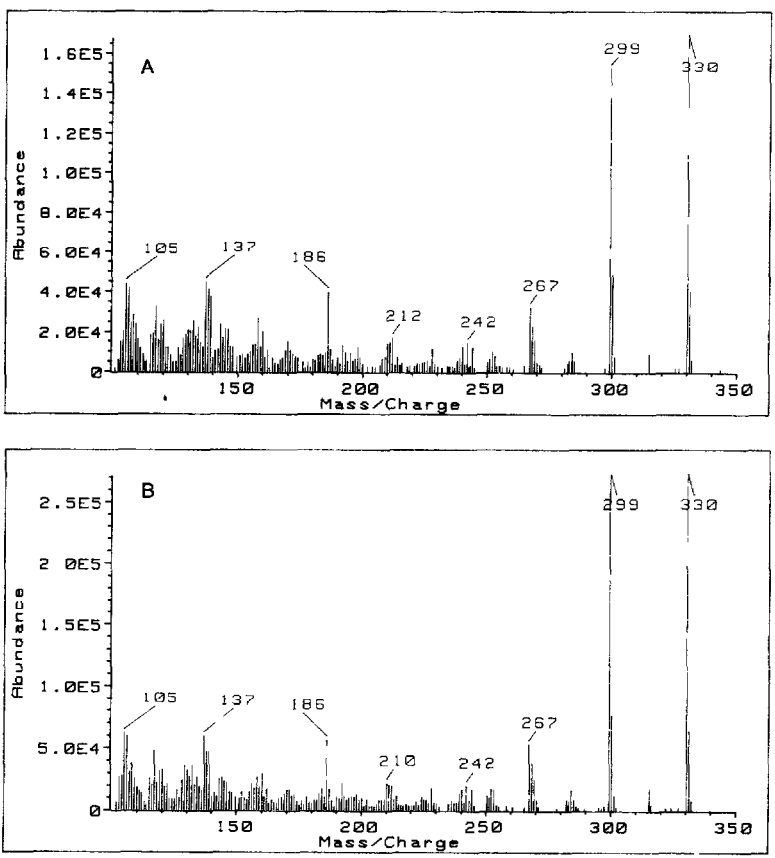

Fig. 2. Mass spectra of the initial major metabolite of nortestosterone in (A) pig hepatocyte cultures and (B) a standard of norandrostenedione.

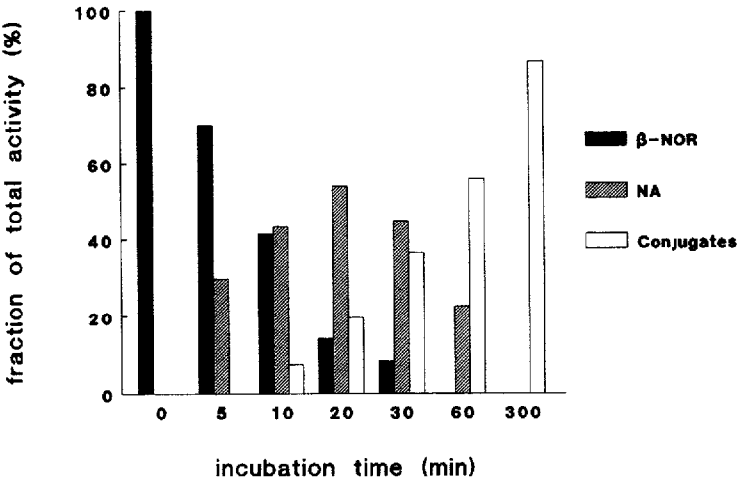

Fig. 3. Relative concentrations of NOR, NA and the 'glucuronide' fraction (retention time 10 min) at different time intervals after initiation of the reaction.

of hepatocytes for biotransformation studies of hormones. Especially the ready use of radiolabelled compounds in in vitro models offers great advantages. Confirmation of in vitro results by in vivo experiments with unlabelled compounds is required but might be relatively simple, following identification of the major in vitro metabolites. Regarding possible species and sex differences in the metabolism of xenobiotics, the need to use hepatocytes isolated from target animals should be stressed.

In addition to biotransformation studies, the use of primary hepatocyte cultures might be helpful in the production of both standards and reference materials necessary for the validation of analytical methods. Further, this model offers the possibility of studying the role of the liver in possible drug–hormone interactions.

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