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Note

Rapid determination of guinea pig hepatic microsomal oestrone-3-sulphate 16α-hydroxylase activity by high-performance liquid chromatography

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Previous work from this laboratory[1] has shown that incubation of oestrone-3-sulphate ($OE_1$3S) with guinea pig (GP) hepatic microsomes leads to hydroxylation at C-16, predominantly in the α configuration. In addition, this activity has been proven to be a cytochrome P_{450}-mediated process[2]. 16α-Hydroxylation of oestrogens is of quantitative significance in man[3,4] and additional evidence suggests that $OE_1$3S may be the primary substrate for oestrogen 16-hydroxylation[5]. These findings have important ramifications since $OE_1$3S is the predominant circulating oestrogen in man[6–8] and has a considerably longer plasma half-life than does the corresponding free steroid, oestrone (OE_1)[9]. Furthermore, 16α-hydroxylation of OE_1 to yield 16α-hydroxyoestrone (16αOH-OE_1) has been found to be of significance in certain pathophysiological states[10–12]. The consequences of 16α-hydroxylation therefore necessitate further research into enzyme regulation and mode of action, and with the finding that 16-hydroxysteroids are the sole products of phenolic steroids when incubated with GP liver[13], the GP hepatic microsomal system presents itself as an ideal model for this detailed study.

In 1980, Tsoutsoulis and Hobkirk[14] developed a radiochromatographic assay for the determination of $OE_1$3S 16α-hydroxylase activity in the guinea pig. This technique, although reliable, was tedious due to its multi-step regimen and complicated the analysis of multiple samples. Other workers[15,16] have illustrated the usefulness of high-performance liquid chromatography (HPLC) for the separation of oestrogen conjugates and thus, in this paper, we report on the integration of HPLC into the 16α-hydroxylase assay and the potential benefits.

MATERIALS AND METHODS

All materials and equipment, unless otherwise indicated, were obtained from Fisher Scientific (Toronto, Canada).

Animals used in this study were immature (< 3 weeks) pigmented guinea pigs

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of the English short hair variety, bred in our own animal quarters. The preparation of microsomes and microsomal incubations were as previously described[2,14] with slight modifications. Incubations were stopped with 2 ml of 3 *M* acetate buffer (pH 4.0) and samples were placed on ice for 15 min. They were then passed through a C_{18} Sep-Pak® cartridge (Waters Scientific, Canada), washed twice with distilled deionized water (2 ml) and steroids eluted with 7 ml of methanol. Samples were taken to dryness under a nitrogen stream and resuspended in 1 ml of methanol. All samples and standards were further processed by filtration through a 0.45-μm Millex® HV filter unit (Millipore, U.S.A.) and stored at −20°C in methanol until required.

The HPLC system was from Waters and consisted of a Model 6000A solvent delivery system, a Model 710 B Wisp® autoinjector, the Waters data module (Model 730) with system controller (Model 720) and a Model 441 UV detector. The columns were 10-μm C_{18} Bondapak® (300 × 4.6 mm) and 5-μm C_{18}-Resolve® (150 × 3.9 mm), connected in series, and 10-μm C_{18} Radial Pak® (5 mm I.D.). All chromatographic operations were performed at room temperature, and the solvents (Baker® HPLC-grade; Canlab, Canada) were filtered through 0.45-μm Durapore® filters (Millipore, U.S.A.) and degassed by sonication prior to use.

The separation of steroid sulphates from microsomal incubations was achieved through isocratic elution in the Bondapak/Resolve system with 0.01 *M* KH_2PO_4–methanol (1:1, v/v) at a flow-rate of 0.5 ml/min. The effluent was collected in vials at 0.5-min intervals on a LKB RediRac® and 5 ml of NEN formula-947 scintillation cocktail (NEN, Canada) were added. Fractions were counted on a Nuclear Chicago Unilux IIA spectrometer and a radiochromatographic profile was obtained. The peak identity was verified by chromatography of authentic [6,7-^{3}H]-17β-oestradiol-3-sulphate ([6,7-^{3}H]-$OE_2$3S) and [6,7-^{3}H]-$OE_1$3S (NEN, Canada). The presence of 16α-hydroxyoestrone-3-sulphate (16αOH-$OE_1$3S) and oestriol-3-sulphate ($OE_3$3S) was verified by solvolysis of the peak thought to contain these metabolites in glacial acetic acid–ethyl acetate (1:9, v/v) at 50°C, overnight[17]. Solvolysed samples, treated as previously described, were then analysed by isocratic elution with 35% (v/v) acetonitrile–water on a C_{18} Radial Pak column at a flow-rate of 1 ml/min. Fractions were collected at 0.2-min intervals and counted. Unlabelled standards, oestriol (OE_3) and 16αOH-OE_1 (Steraloids, U.S.A.), were run concurrently and their elution profile, monitored at 254 nm, was compared with the radiochromatographic profile of the solvolysed sample.

RESULTS AND DISCUSSION

Fig. 1A illustrates the typical radiochromatographic profile of the oestrogen sulphates derived from microsomal incubations. Incubations done in the presence of reduced cofactor (NADPH) led to the appearance of three major peaks (I, II and III). In the absence of NADPH, the control profile consisted of a single peak (II) with a retention time (26.0 min) corresponding to that of $OE_1$3S (Fig. 1B). Peak III appeared in the presence of NADPH and corresponded well with $OE_2$3S of the standard profile (Fig. 1B), confirming the involvement of reductases[1] in the assay system.

Due to the lack of appropriate oestrogen sulphate standards, peak I (Fig. 1A) could not be directly identified chromatographically; it was therefore solvolysed and

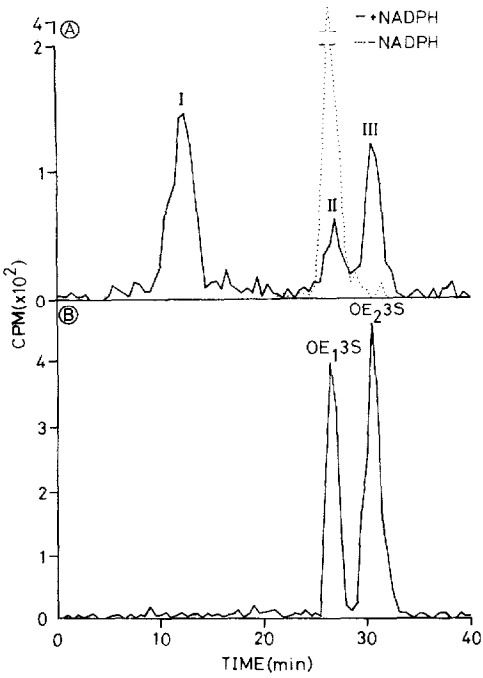

Fig. 1. HPLC analysis of oestrogen sulphate metabolites on a C_{18} Bondapak column and a C_{18} Resolve column connected in series. A, Radiochromatographic profile of oestrogen sulphates from microsomal incubations in the presence and absence of NADPH; B, radiochromatographic profile of $OE_1$3S and $OE_2$3S.

its elution profile (Fig. 2A) compared with that of unconjugated 16α-hydroxylated oestrogen standards (Fig. 2B). Comparison of the two profiles shows that peaks IV and V correspond to OE_3 and 16αOH-OE_1, respectively. The prevalence of the reduced product, OE_3, again confirms the involvement of reductases and is in agreement with work done in man[18] suggesting that 16αOH-OE_1 is the probable precursor of OE_3. Thus, the major components of the radiochromatographic profile (Fig. 1A) and their probable routes of production can be summarized as in Fig. 3.

The chromatographic method, as described, provides significant advantages for the quantitative analysis of $OE_1$3S 16α-hydroxylase. With this technique, there is a well defined separation between the starting compound ($OE_1$3S) and the 16α-hydroxylated metabolites of interest (peak I) and activity can be directly expressed as a per cent of total radioactivity with recoveries consistently >97%. The assay system also offers a significant reduction in time over previously available methods[2,14,19] which took, on the average, 3 days to perform. Furthermore, the latter systems consisted of a multi-step regimen with numerous points at which losses and errors could occur. Incorporation of HPLC increases the potential for improved recoveries and greater precision due to decreased handling of the sample. It also provides the capacity to automate analysis which is of particular importance when dealing with the study of endogenous and exogenous factors (hormones and drugs, respectively) and their effect on the oestrogen 16α-hydroxylase activity[2].

Little has been published on the quantitation of 16αOH-OE_1[20–22] and on the

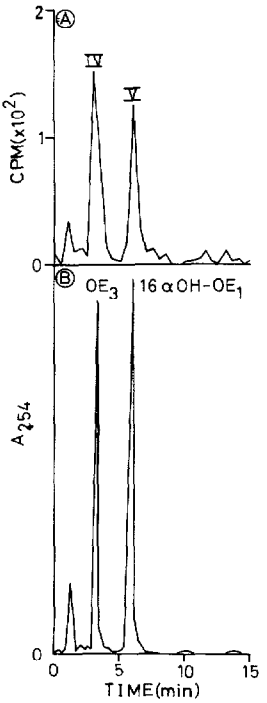

Fig. 2. HPLC analysis of unconjugated oestrogens on a C_{18} Radial Pak column. A, Radiochromatographic profile of solvolysed peak I (Fig. 1A); B, elution profile of unconjugated oestrogen standards as monitored at 254 nm; OE_3 and 16αOH-OE_1.

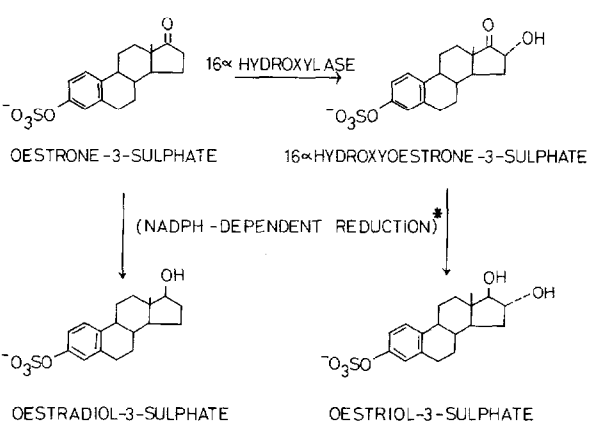

Fig. 3. Major oestrogen sulphate metabolites arising from incubation of OE_13S with GP hepatic microsomes in the presence of NADPH. The process marked with an asterisk is true only in that controls, in the absence of NADPH, failed to elicit reduced products.

HPLC properties of this compound[23]. Thus, the technique described here or modifications thereof may lend itself towards a better understanding of the physiological role of 16αOH-OE_1.

In conclusion, an HPLC method for the determination of GP hepatic $OE_1$3S 16α-hydroxylase activity has been presented. Although it lacks the resolution of previously published methods[15,16], the separation of the oestrogen sulphates derived from microsomal incubations is in accordance with the practical application of quantitating enzymatic activity. Finally, this technique is more rapid, concise and has greater potential capacity.

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