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MICROSOMAL GLUCURONIDATION OF SELECTED STEROIDS USING A RAPID RADIOMETRIC ASSAY

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SUMMARY

Glucuronidation of testosterone, estrone, estradiol and estriol was investigated in rat liver microsomes using a rapid radiometric method. The method is one-step with activity measured in the same vial in which the reaction is conducted. Unreacted substrate is partitioned from the aqueous incubation medium into the non-polar scintillation fluid. Glucuronides remain in the aqueous fraction and therefore, are not detected by liquid scintillation counting which enables one to establish the glucuronidation rate by following substrate disappearance. This rapid method is less variable than more time-consuming extraction procedures and can be used easily to obtain kinetic data. Parameters were established for reaction linearity with respect to time and the addition of microsomal protein. Male rat liver microsomal glucuronyltransferase conjugated testosterone (2·6 nmol/min/mg protein) most rapidly followed by β-estradiol, estrone, and estriol, respectively. Kidney and lung microsomes had no detectable steroid glucuronyltransferase activity, although these tissues had significant levels of p-nitrophenol glucuronyltransferase.

INTRODUCTION

UDP glucuronyltransferases are considered to be important enzymes for the regulation of many steroid hormones and they are also active in the detoxication of many xenobiotics [1–3]. There are many fairly rapid colorimetric methods that have been used to measure glucuronidations of foreign compounds such as p-nitrophenol [4] and phenolphthalein [5]. A radioassay for 1-naphthol and some other substrates used in this laboratory has been described recently [6]. However, present methods used to determine steroid glucuronidations are more difficult and they involve extraction steps which are quite time consuming and often have high experimental variations.

In this paper, we report a simple one-step procedure for determining steroid glucuronyltransferase activity based on the partition of unreacted substrate into non-aqueous scintillation fluid. This method represents a modification of the procedure that was used previously to measure 1-naphthol glucuronidation and data was presented to indicate that steriod glucuronidation measurements could possibly be made by a one-step radioassay [6].

Kinetic and other enzyme parameters are derived using the rapid method and direct comparisons are made with other procedures used to measure steroid glucuronyltransferase. Investigations reported here compare microsomal steroid glucuronidations in different tissues as well as glucuronidation rates of several substrates; testosterone, estrone, estradiol and estriol.

MATERIALS AND METHODS

Animals

Male Charles River rats (8 weeks old, approx. 250 g) were used in these studies. Liver, lung and kidney microsomes were prepared as described previously [7] and resuspended in 150 mM Tris–HCl buffer (pH 7·4 at 4°C) so that 1·0 ml suspension contained microsomes from 0·5 g tissue (wet wt.). Microsomal suspensions were mixed throughly with Triton X-100 (0·25 μl/mg microsomal protein). This concentration of Triton X-100 yielded optimum enzyme activity.

Chemicals

Steriod substrates were testosterone, estrone, estradiol and estriol. Radioactive steroids were [1,2,6,7-^{3}H]-testosterone (80–105 Ci/mmol), [4-^{14}C]-testosterone (50–60 mCi/mmol), [6,7-^{3}H]-estrone (40–60 Ci/mmol), [6,7-^{3}H]-estradiol (40–60 Ci/mmol), [4-^{14}C]-estriol (45–60 mCi/mmol), [1,2-^{3}H]-corticosterone (40–60 Ci/mmol), [7-^{3}H]-dehydroepiandosterone (10–

25 Ci/mmol), [1,2-^{3}H]-testosterone-β-D-glucuronide (40–60 Ci/mmol, [6,7-^{3}H]-estrone-β-D-glucuronide (40–60 Ci/mmol, and [6,7-^{3}H]-estradiol-17β-D-glucuronide (40–60 Ci/mmol).

Assay methods

The incubation system for glucuronidation measurements was added to liquid scintillation vials and consisted of the following: 1·2 ml 75 mM Tris–HCl buffer (pH 7·4), 1·0 μmol UDPGA, 10·0 μmol $MgCl_2$, 0·3 μmol unlabeled substrate in 50 μl methanol and 1×10^5 d.p.m. labeled substrate in 50 μl methanol. This volume of methanol was used to insure substrate solubilization and had no apparent effect on glucuronyltransferase activity. The incubation contents were warmed at 37°C for 3 min and then 0·4–0·6 mg microsomal protein was added. The volume of the medium was 1·3 ml. The incubation period was 10 min when measuring the conjugation of testosterone and 20 min when measuring conjugation of the other substrates because the testosterone glucuronidation rate is more rapid. The reaction was stopped by the addition of 10 ml non-aqueous scintillation fluid prepared by mixing 43 ml liquiflour (New England Nuclear)/l toluene. Samples were capped, shaken for 10 sec on a vortex mixer, and radioactivity counted in the same vials in which the incubation reactions were performed. Addition of the toluene-based scintillation fluid resulted in a two-phase mixture (toluene on top and the aqueous fraction on the bottom). Unreacted substrate partitioned into the toluene and this radioactivity was detected in a Packard Tri-Carb liquid scintillation counter equipped with an Automatic Quench Analyzer. Glucuronides remained in the aqueous fraction and since ^{14}C and ^{3}H in a water medium do not scintillate, radioactivity associated with glucuronides is not detectable by liquid scintillation spectrometry. This phenomenon enables steroid glucuronidation rates to be measured by substrate disappearance. Blank values were obtained by omitting UDPGA from the reaction mediums. The incubation blanks represent 0% activity and correct for the amount of substrate remaining in the aqueous fraction (incubation medium). Glucuronides detected after addition of scintillation fluid to incubation mediums reflect the amount of radioactivity detected after 100% glucuronidation of substrate. Enzyme activity using 300 nmol substrate in the incubation medium is expressed by the equation:

$$1 - \left[\frac{\begin{matrix}\text{radioactivity} \\ \text{after incubation}\end{matrix} - \begin{matrix}\text{radioactivity} \\ \text{after incubation}\end{matrix} \times \begin{matrix}\text{Fraction of respective } \beta\text{-D} \\ \text{glucuronide detected}\end{matrix}}{\text{Radioactivity in blank}}\right] \times 300\,\text{nmol} = \text{nmol substrate conjugated.}$$

An example for calculating testosterone glucuronidation in which 7×10^4 d.p.m. are detected is as follows:

$$1 - \frac{7 \times 10^4 - (7 \times 10^4)(0{\cdot}04)}{\frac{26{\cdot}1}{27{\cdot}1}(10^5)} \times 300 = 90{\cdot}9 \text{ nmol conjugated.}$$

Further demonstration of the validity of this assay method is given in the results section.

The rapid method was compared to a slightly modified method [8] of Rao *et al.* [9–11] involving butanol extraction of glucuronides. The incubation contents and reaction conditions were the same as described above for the rapid assay and the reaction was stopped by the addition of 5 ml ethyl acetate. We observed no difference in reaction rate whether methanol or propylene glycol [8–11] was used to solubilize substrates.

Product formation was also measured by cooling the samples to −10°C after the reaction was stopped with non-aqueous scintillation fluid. The water phase froze but the toluene fraction remained liquid. This allowed the toluene fraction, containing the substrate, to be decanted whereas the glucuronidated steroids remained in the water fraction. Ten milliliters of aqueous scintillation fluid (5·0 g PPO, 0·3 g PPOPOP/l toluene containing 300 ml Triton X-100) was then added to the water fraction and the amount of glucuronides was detected by liquid scintillation counting.

To verify the above described assay methods, steroid aglycones were isolated from their respective glucuronides by chromatography of 1·0 ml of the incubation mixture on DEAE-cellulose columns [8, 12].

Microsomal protein was quantified by the method of Lowry *et al.* [13].

RESULTS AND DISCUSSION

Partition coefficients of selected radioactive steroids and their respective β-D-glucuronides into the scintillation fluids are presented in Table 1. These data demonstrate that testosterone, estrone, estradiol, corticosterone and dehydroepiandosterone are readily extractable into non aqueous scintillation fluid from incubation mixtures (only UDPGA was omitted). On the other hand only small amounts of the β-D-glucuronides tested were extractable (less than 5% in the three glucuronides tested). High partition of the agly-

Table 1. Partition coefficients of some radioactive steroids and their respective β-D-glucuronides into non-aqueous scintillation fluid from incubation mediums*

Steroid	Aglycone	β-D-Glucuronide
[^{3}H]-Testosterone	26·1 ± 1·1	0·04 ± 0·01
[^{14}C]-Testosterone	24·6 ± 0·7	—
[^{3}H]-Estrone	20·3 ± 2·0	0·05 ± 0·02
[^{3}H]-Estradiol	10·1 ± 0·4	0·03 ± 0·02
[^{14}C]-Estriol	0·7 ± 0·1	—
[^{3}H]-Dehydroepiandosterone	84·2 ± 1·2	—
[^{3}H]-Corticosterone	13·9 ± 0·9	—

* Incubation mediums were as described in the Materials and Methods for blanks (minus UDPGA). Each value represents the mean ± S.D. derived from six incubations using male liver microsomes. Partition coefficients derived by; amount partitioning into non-aqueous scintillation fluid divided by amount remaining in the aqueous fraction. Partition coefficients measured at 4°C (temperature of liquid scintillation counter).

cone into toluene compared to partition of the glucuronide indicates suitability of this assay method for a particular compound. Estriol was the only steroid tested that does not appear to be well suited for the rapid glucuronyltransferase assay.

Mixed function oxidase reactions, particularly hydroxylations, were thought to possibly influence steroid glucuronidation rates by changing the number of available glucuronidation sites. To test this idea, testosterone glucuronidation rates were determined under oxygen, nitrogen and carbon monoxide atmospheres. Incubations conducted under oxygen would maximize mixed function on oxidase activity and those conducted under carbon monoxide would inhibit oxidative activity due to the formation of the CO-cytochrome P-450 complex [14]. Results show that testosterone glucuronidation rates were only slightly different under all of the atmospheric conditions tested (Table 2). This information suggests that glucuronidation of testosterone occurred only at the 17 position or the glucuronidation rate of testosterone was unaffected by hydroxylations, if they did occur. To check further if glucuronidation was occurring at sites other than the 17 position, microsomal incubations were chromatographed on DEAE-cellulose columns. Only one glucuronide peak was resolved and this co-chromatographed with a known standard of testosterone β-D-glucuronide (Fig. 1).

To determine whether the assay method was affected by tritium exchange of substrate with components of the incubation medium, including microsomes, glucuronidation rates of [4-^{14}C]-testosterone and [1-2-^{3}H]-testosterone were compared. Results revealed that glucuronidation rates were the same using either isotope.

Table 2. Testosterone glucuronidation rates under oxygen, nitrogen and carbon monoxide atmospheres*

(nmol Glucuronidation/min/mg protein)		
Oxygen	Nitrogen	Carbon monoxide
4·72 ± 0·35	4·92 ± 0·36	5·51 ± 0·18

* Male rat liver microsomes used as the enzyme source and each value represents the mean ± S.D. derived from six incubations.

Reaction linearities with respect to time and with respect to the addition of microsomal protein for testosterone glucuronidation are illustrated in Figs. 2 and 3. Glucuronidation was linear with addition of protein up to 1·0 mg using a 10 min incubation period. Reaction rates exhibited linearity for up to 20 min using 0·5 mg microsomal protein. In general, linearity can be expected up to 30% glucuronidation of testosterone. When glucuronidation exceeds 30%, under the specified incubation conditions, substrate and/or UDPGA depletion apparently act to decrease the reaction rate.

Kinetic data for liver microsomal estrone and testosterone glucuronidations were obtained under saturating concentrations of UDPGA. The K_m was $9{\cdot}6 \times 10^{-5}$ M for testosterone and $6{\cdot}2 \times 10^{-5}$ M for estrone. Estrone and testosterone, when incubated together, inhibited the glucuronidation rates of each other (Table 3), suggesting that the same enzyme is conjugating both substrates in the microsomes. However, cytosol steroid glucuronyltransferases appear to exhibit multiplicity [10, 15].

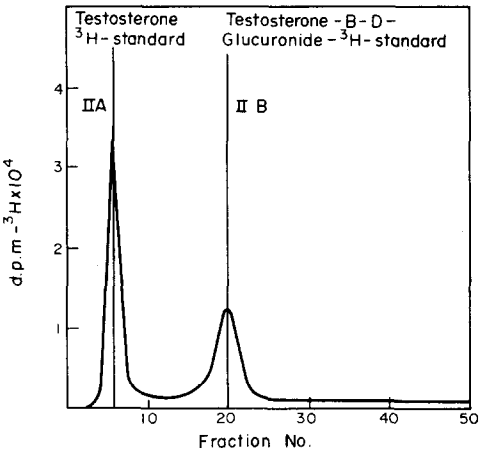

Fig. 1. DEAE-cellulose gradient elution profile of the microsomal incubation for measuring [^{3}H]-testosterone glucuronidation. The elution buffer started with 0·1 M Tris–HCl and ended with 0·05 M Tris–HCl (pH 7·5) using a total vol. of 600 ml. The incubation mixture was as described in the Materials and Methods. Vertical solid lines represent chromatographic locations of standards as indicated.

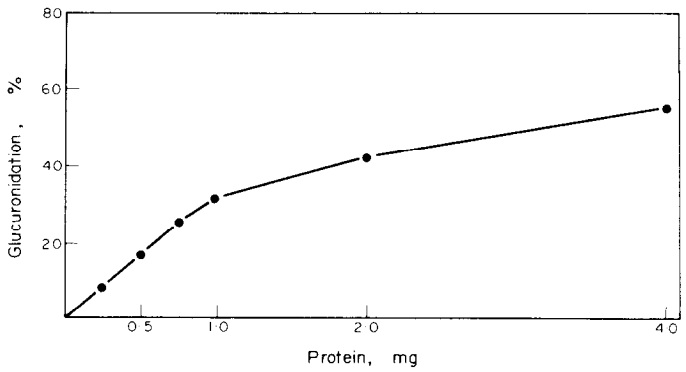

Fig. 2. Percentage glucuronidation of [^{3}H]-testosterone with respect to the addition of microsomal protein. The concentration of testosterone was 0·4 mM and the incubation period was 10 min.

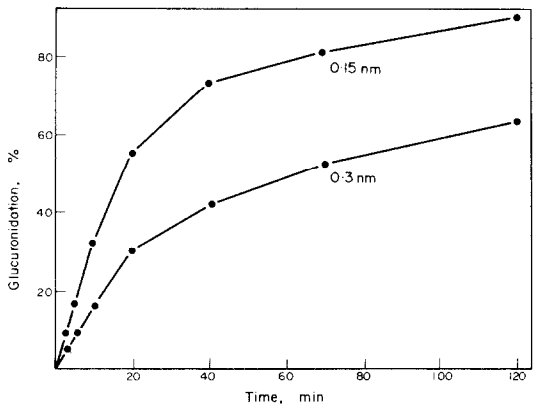

Fig. 3. Percentage glucuronidation of [^{3}H]-testosterone with respect to time. Testosterone concentrations were either 0·15 or 0·30 mM and 0·5 mg microsomal protein was used in the incubation medium.

Comparative rates for liver microsomal glucuronidation of testosterone, estrone, estradiol, and estriol are presented in Table 4. Male liver microsomes glucuronidated testosterone most rapidly followed by estradiol, estrone and estriol, respectively. Estradiol might be conjugated at two sites [16, 17] and estriol might be conjugated at three sites [16, 18, 19]. However, these assay methods measure only total glucuronidation rates and do not differentiate the site of reaction. Data are given for the results obtained by the rapid method as well as those obtained using the butanol extraction method [9–11]. Testosterone glucuronidation rates were also measured by resolving substrate from product on DEAE–cellulose columns and by measuring radioactive glucuronides in the water phase. Values for the rapid method were similar

Table 3. Glucuronidation rates of testosterone and estrone, alone or in combination, by rat liver microsomes

(nmol Glucuronidation/min/mg protein)			
Testosterone*		Estrone*	
Alone	+ Estrone	Alone	+ Testosterone
3·42 ± 0·27	1·98 ± 0·25	1·45 ± 0·12	0·83 ± 0·07

* The concentration of each steroid, alone or in combination, was 0·3 mM. Each value represents the mean + S.D. derived from six incubations.

Table 4. Steroid glucuronidation rates measured by the rapid radioassay, the butanol extraction method or the isolation of product on DEAE–cellulose columns

	(nmol Glucuronidation/min/mg protein)			
Substrate	Rapid*	Butanol extraction†	DEAE-cellulose‡	Radioactivity in the water phase§
Testosterone	2·63 ± 0·06	2·19 ± 0·32	2·53	2·54 ± 0·07
Estrone	0·83 ± 0·04	0·66 ± 0·07		—
Estradiol	0·90 ± 0·07	0·87 ± 0·09		
Estriol	0·37 ± 0·08	0·26 ± 0·08		—

* The rapid method conducted as described in the Materials and Methods.
† The modified method [8] of a previously-used procedure [9–11].
‡ Product resolved from substrate on DEAE-cellulose columns [8,12].
§ Product formation measured as described in the Materials and Methods by toluene extraction of substrate and subsequent measurement of radioactivity in water phase.

to the extraction and DEAE-column methods. In general, data obtained by the rapid method is more reproducible, presumably due to it being only a one-step procedure. Accuracy of both methods were reassured by the positive comparision with rate data obtained by measuring product formation on DEAE–cellulose columns and in the water phase after toluene extraction. Sensitivity of the rapid method, using 0·3 mM substrate, is approximately 0·1 nmol conjugated/min/mg protein. The sensitivity, due to the nature of the radioactive method, could be increased by decreasing substrate concentration.

p-Nitrophenol is readily conjugated by rat kidney glucuronyltransferase [20]. However, we detected no glucuronidation of testosterone nor estrone using the same microsomal preparations that actively glucuronidate p-nitrophenol. Lack of steroid enzyme activity in kidney microsomes was verified by DEAE–cellulose chromatography. This information provides more evidence for the contention that different glucuronyltransferases conjugate steroids than conjugate p-nitrophenol and other more exogenous-type substrates [8, 15].

In summary, we feel that the rapid method described here for measuring steroid glucuronidation provides many advantages over previously-used procedures. The greatest advantage is the rapidity of the new assay coupled with increased accuracy. Other advantages are: (1) applicability to a wide variety of substrates; (2) β-glucuronidase activity of steroids can be determined by the use of the reverse method (partition of product into scintillation fluid); and (3) the partition factor over time is stable so that samples can be stored before counting.

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