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Sensitive liquid chromatographic method using fluorescence detection for the determination of estradiol 3- and 17-glucuronides in rat and human liver microsomal incubations: formation kinetics

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Abstract

We have developed a sensitive and specific HPLC-fluorescence assay for the determination of estradiol-3-glucuronide and estradiol-17-glucuronide in human and rat liver microsomal incubations. The method utilizes a mobile phase comprised of acetonitrile and 50 m*M* ammonium phosphate buffer (35:65, v/v) that is pumped though a phenyl column at 1 ml/min; the run time is less than 15 min. Calibration curves for both metabolites were linear over the range 20–4000 pmol. The intra- and inter-day coefficients of variation were <6%. In both rat and human liver microsomes, the formation of estradiol-3-glucuronide displayed atypical kinetics (consistent with activation), while estradiol-17-glucuronide formation was consistent with classical Michaelis–Menten kinetics. Overall, the assay described is a sensitive and reproducible method for the determination of estradiol glucuronides in liver microsomal preparations. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Formation kinetics; Estradiol glucuronide

1. Introduction

Glucuronidation is an important process in maintaining the normal physiological concentration of many vital endogenous substances such as bilirubin, bile acids, steroids, and thyroid hormones [1]. Estradiol-17β (Fig. 1) is a naturally occurring steroid hormone and is the most active endogenous estrogen. Previous studies have shown that the hydroxy groups at positions 3 and 17 undergo glucuronidation by at

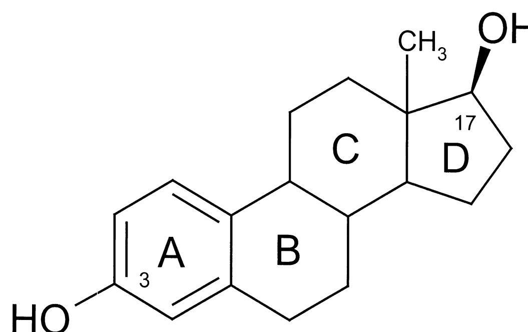

Fig. 1. Chemical structure of estradiol.

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least two different uridine 5′-diphosphate [UDP]-glucuronosyltransferase (UGT) isoenzymes. The conjugation of the phenolic 3-OH of estradiol and its synthetic congener, ethinylestradiol, has been reported to be mediated mainly by UGT1A1 in humans [2,3]. The glucuronidation of the 17β-OH is thought to be mediated by multiple UGTs including UGT2B1 and UGT2B3 in rats [4,5]. Recently, it was observed that human UGT2B7 is capable of glucuronidating the 17β-OH of estradiol [6].

Different analytical techniques have been used to measure the rate of formation of estradiol conjugates in microsomal incubations. These methods have generally involved measuring the radioactivity of a labeled substrate or co-substrate (i.e. UDPGA) following extraction of the parent drug or UDPGA from the metabolites with liquid–liquid extraction [7,8]. Determination of estradiol 3-OH and 17-OH conjugates has also been achieved by solid-phase extraction with subsequent gradient elution and UV detection [3]. More recently, a gradient high-performance liquid chromatography–mass spectrometry (HPLC–MS) method was used to quantify both estradiol conjugates in human liver microsomes [9]. Although these techniques are suitable for the measurement of estradiol glucuronides, a non-radioactive assay that does not require extraction or MS detection was desired. Thus, the aim of the current study was to develop a simple, sensitive and highly specific HPLC-fluorescence assay for the determination of estradiol glucuronides in rat and human liver microsomes.

2. Experimental

2.1. Reagent and chemicals

β-Estradiol, β-estradiol-3-(β-D-glucuronide) [E-3-G], β-estradiol-17-(β-D-glucuronide) [E-17-G], bilirubin, alamethicin, uridine diphosphoglucuronic acid (UDPGA), magnesium chloride, and dextromethorphan were purchased from Sigma (St. Louis, MO, USA). Potassium phosphate, ammonium phosphate, ethanol, and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals and solvents were of the highest grade available.

Male Sprague–Dawley rats (200–250 g) were purchased from Harlan Sprague–Dawley Farms (Indianapolis, IN, USA). Animals were allowed water and food ad libitum and maintained in accordance with standard recommended procedures. Immediately prior to sacrifice, animals were anesthetized with intraperitoneal ketamine/xylazine and the liver was quickly harvested. Human liver samples were obtained from patients undergoing partial hepatectomy for hepatic metastatic cancer after informed consent; the protocol was approved by the local Institutional Review Board. Liver samples were carefully removed from the unaffected area adjacent to the resected tumor and were normal by both macroscopic and microscopic examinations. Microsomes were prepared from whole tissue homogenate by differential centrifugation and stored at −80 °C until use. Protein concentration was quantified using the method of Lowry [10].

2.2. Instrumentation

The HPLC system consisted of a model 717 autosampler, a model 501 HPLC pump, and a model 470 fluorescence detector set at λ_{EX} of 210 nm and λ_{EM} of 300 nm from Waters (Milford, MA, USA). The UV spectrum of both E-3-G and E-17-G showed maximum absorbance at approximately 210 nm and a scan conducted at a fixed λ_{EX} of 210 demonstrated maximum emission at 300–310 nm for E-3-G and E-17-G. Dextromethorphan was used as the internal standard since it has fluorescent and chromatographic properties similar to estradiol and its conjugates. The mobile phase consisted of 35% acetonitrile and 65% 50 m*M* ammonium phosphate buffer (pH 3). This was pumped at a flow-rate of 1 ml/min through an Alltima phenyl column, 5 μ, 4.6×250 mm (Alltech Associates, Deerfield, IL, USA). Signal output was captured using Millennium[32] software, version 3.05 (Waters, Milford, MA, USA).

2.3. Stock solutions

A stock solution of 0.1 *M* potassium phosphate (pH 7.1) buffer was prepared in deionized water. UDPGA (50 m*M*) and magnesium chloride (5 m*M*) were prepared in phosphate buffer solution. Alamethicin (200 ng/μl) was prepared in phosphate

buffer containing 10% ethanol. A 50 m*M* ammonium phosphate buffer (pH 3, adjusted with phosphoric acid) was prepared in deionized water. For calibration standards, separate stock solutions (1000 pmol/μl) of E-3-G and E-17-G were prepared in methanol and further diluted with methanol to final concentrations of 10 and 100 pmol/μl (1 pmol=448 pg). Dextromethorphan (internal standard) was also prepared in methanol at a final concentration of 5 ng/μl. Standards were prepared in incubation buffer containing all incubation constituents except microsomes (preliminary experiments showed that E-3-G and E-17-G recoveries were not affected by the presence of microsomal protein).

2.4. Estradiol incubation and HPLC assay

Rat or human liver microsomes (0.5 mg/ml final protein concentration) and alamethicin (30 μg/mg protein) were pre-incubated on ice for 5 min. The incubation mixture consisted of magnesium chloride (1 m*M*), and estradiol (over a 1 to 150 μ*M* concentration range). Final incubation volume was 250 μl. Preliminary experiments demonstrated formation rate linearity up to 90 min and over a range 0.25–1.5 mg/ml of protein under the conditions tested. UDPGA concentrations of 6 m*M* for human microsomes and 10 m*M* for rat microsomes were based on preliminary experiments, which indicated that these concentrations are saturating (apparent K_m's of 2.1 and 3.9 m*M*, respectively). Alamethicin was used to remove the known latency of UGTs, since we and others have recently shown that alamethicin is more efficient than the commonly used detergents (e.g. Brij-58) in maximizing UGT activity in microsomal incubations [9,11].

The reaction was started by adding UDPGA and placing the tubes in a 37 °C water bath. Blank incubations were performed without UDPGA or substrate. After 60 min of incubation, the reaction was terminated by adding 25 μl of 6% perchloric acid and 20 μl of dextromethorphan (5 ng/μl) as internal standard (Int. Std.). Samples were vortex-mixed, cooled on ice, and then centrifuged at 2000 *g* for 5 min. The resulting supernatant was transferred into autosampler vials and 75 μl was injected for HPLC analysis.

2.5. Calibration and linearity

Calibration curves were obtained daily for 3 days using standards containing six different amounts of E-3-G and E-17-G (20, 100, 500, 1000, 2000, and 4000 pmol per 250 μl). Standards were prepared in incubation buffer (250 μl) by the addition of 4–20 μl of the methanol stock solutions as above. At the standard amounts specified, 5 pmol to 1000 pmol (2.2–448 ng) of E-3-G and E-17-G were injected on column with a 75 μl injection. Curves were constructed by calculating the peak-height ratios of E-3-G or E-17-G to that of the Int. Std. Calibration curve data points were fit using linear regression analysis with $1/y^2$ weighting.

2.6. Precision and accuracy

The precision and accuracy of the assay was determined using quality control (QC) samples of known E-3-G or E-17-G amounts (i.e. 50 and 3000 pmol), which were prepared fresh each validation day as described for calibration curve standards. Six replicates of each QC were analyzed on 3 days and the intra- and inter-assay means, standard deviations and coefficients of variation (C.V.) were calculated.

2.7. Stability testing–estradiol glucuronide conjugates

The stability of E-3-G and E-17-G was tested using 10 replicates of QC samples (50 and 3000 pmol), which were injected immediately after sample preparation and then again after 24 h in the autosampler at room temperature. Comparison of the calculated amounts of E-3-G and E-17-G provided a measure of metabolite stability under normal operating conditions.

2.8. Data analysis

Descriptive statistics (mean, SD, C.V.) were calculated using Microsoft Excel. The apparent kinetic parameters of K_m, V_{max} and *n* (Hill constant, where appropriate) were determined by non-linear regression analysis (Prism 3.0, GraphPad software, San Diego, CA, USA). Data were fit to the Michaelis–Menten equation (E-17-G) or to the Hill equation

(E-3-G). Model discrimination was guided by visual inspection, the sum of the squares of the residuals, and the standard errors of the parameter estimates.

3. Results

3.1. Chromatographic method

Representative chromatograms of precipitated microsomal incubations are shown in Figs. 2 and 3. E-3-G, E-17-G, Int. Std., and estradiol were separated within 14 min. The retention times for E-3-G, E-17-G, Int. Std., and estradiol were approximately 4.8, 5.3, 6.4, and 12.2 min, respectively. E-3-G and E-17-G were stable in processed microsomal incubation mixtures for at least 24 h at room temperature. Standard curves for E-3-G and E-17-G were linear over the range 20–4000 pmol (5–1000 pmol on column); the mean correlation coefficient (r^2) for the standard curves was >0.99 (Table 1). The calculated intra- and inter-day C.V.s for E-3-G and E-17-G QC samples were less than 6% with a measured difference from the nominal concentration of less than 4% in all cases (Table 2).

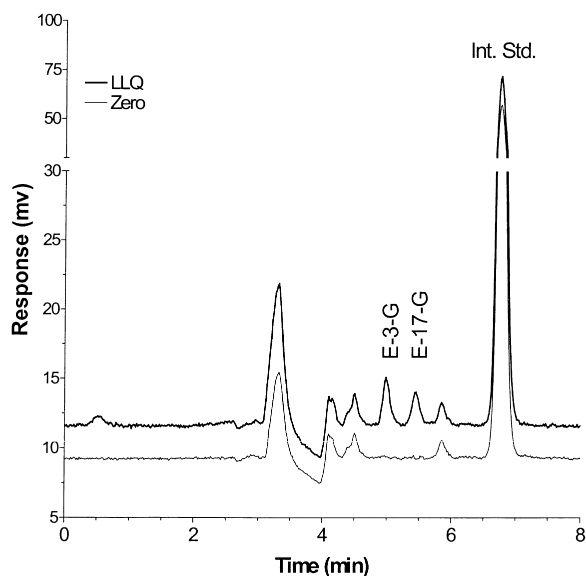

Fig. 2. Representative chromatograms (offset) of incubation buffer spiked with the internal standard only (zero E-3-G and E-17-G), and incubation buffer spiked with 20 pmol (lower limit of quantitation, LLQ) of E-3-G and E-17-G and the internal standard.

Table 1
Linear regression analysis of estradiol-3-glucuronide and estradiol-17-glucuronide calibration curves

Estradiol glucuronide	Slope ($\times 10^{-3}$)	Intercept ($\times 10^{-3}$)	R^2
E-3-G	3.37	8.2	0.998
	3.51	5.3	0.998
	3.49	2.0	0.999
Mean±SD	3.46±0.07	5.2±3.1	0.998±0.001
C.V.%	2.2%		
E-17-G	1.76	0.8	0.997
	1.82	4.9	0.997
	1.79	8.3	0.996
Mean±SD	1.79±0.03	4.7±3.8	0.997±0.001
C.V.%	1.7%		

3.2. Estradiol glucuronidation kinetics

The estimated kinetic parameters for E-3-G and E-17-G in rat and human liver microsomes are presented in Table 3. As shown in Fig. 4, the formation of E-3-G in both rat and human liver microsomes displayed atypical kinetics (hooked Eadie–Hofstee plot, Fig. 4) consistent with activation, while E-17-G formation determined in the same samples was consistent with typical Michaelis–Menten kinetics. Data for E-3-G were best fit to the Hill equation and data for E-17-G were best fit to the Michaelis–Menten equation.

4. Discussion

We have developed a rapid and sensitive HPLC-fluorescence assay for the determination of estradiol glucuronides in rat and human liver microsomal incubations. To the best of our knowledge, this is the first detailed report of a validated method utilizing HPLC-fluorescence for measuring E-3-G and E-17-G in microsomal incubations. The method is reproducible with intra- and inter-assay coefficients of variation of less than 6%. The assay was able to detect very low amounts of E-3-G and E-17-G (i.e. 20 pmol) in the incubation medium.

The utility of the method was demonstrated by

Table 2
Intra- and inter-assay precision and accuracy for estradiol-3-glucuronide and estradiol-17-glucuronide in incubation buffer

Estradiol glucuronide	Amount added (pmol)	Amount found (Mean±SD, pmol)	C.V. (%)
E-3-G			
Intra-day	50	52±2	3.4
(n=6)	3000	2999±59	2.0
Inter-day	50	51±2	3.0
(n=18)	3000	3026±69	2.3
E-17-G			
Intra-day	50	54±3	5.0
(n=6)	3000	2945±28	0.9
Inter-day	50	51±3	5.7
(n=18)	3000	2877±65	2.3

Table 3
Kinetic analysis of estradiol glucuronide formation in rat and human liver microsomes

Estradiol glucuronide	V_{max}^{app} (pmol/min per mg) (Mean±SE)	K_m^{app} (μM) (Mean±SE)	Hill coefficient (n) (Mean±SE)
E-3-G			
Rat	588±43	33±5	2±0.40
Human	497±17	20±2	2±0.14
E-17-G			
Rat	676±45	23±5	N/A
Human	78±1	8±0.4	N/A

measuring estradiol glucuronides in rat and human liver microsomal incubation mixtures. Consistent with previous reports, the formation of E-3-G showed an atypical kinetic profile consistent with autoactivation kinetics (i.e. an allosteric effect), while the formation of E-17-G showed a linear Eadie–Hofstee plot for both rat and human liver microsomes (Fig. 4). This observation is consistent with the recent work of Fisher et al. [9] examining estradiol glucuronidation in human liver microsomes.

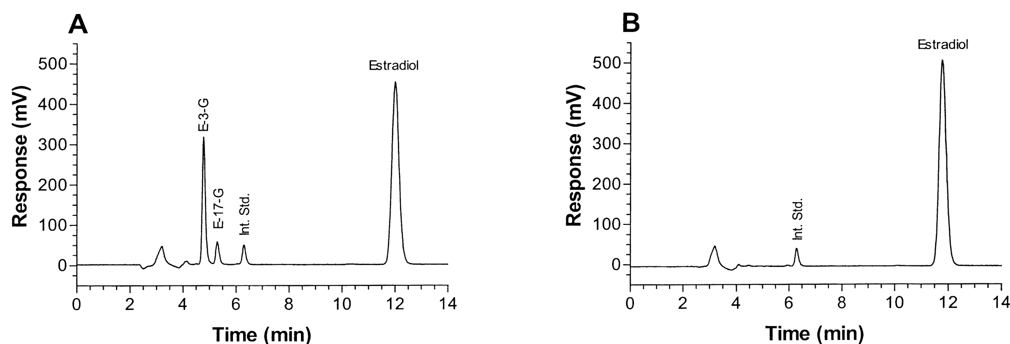

Fig. 3. Representative chromatograms of estradiol, E-3-G, and E-17-G in precipitated human liver microsomal incubation mixtures. Panel A - microsomal incubation containing 150 μM estradiol and 6 mM UDPGA. Panel B - microsomal incubation mixture in the absence of UDPGA. The calculated levels of E-3-G and E-17-G were 3472 and 540 pmol, respectively.

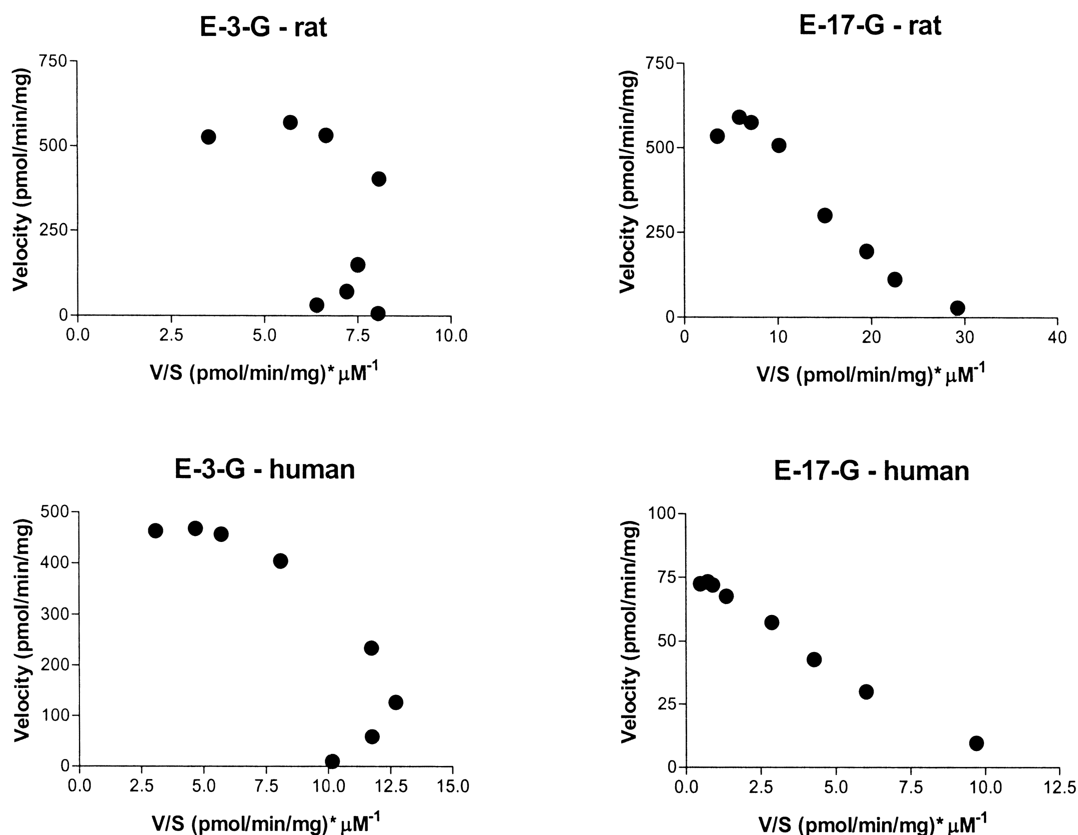

Fig. 4. Eadie–Hofstee plots of estradiol-3-glucuronide and estradiol-17-glucuronide formation in incubations with human and rat liver microsomes.

In summary, a simple and sensitive HPLC method for the direct analysis of E-3-G and E-17-G in rat and human liver microsomal incubations was developed and validated. The method is suitable for use in the in vitro study of UGT-mediated estradiol biotransformation.

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