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Short communication

Separation of testosterone metabolites in microsomal incubates using a new capillary electrophoresis assay

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Abstract

A capillary electrophoresis method has been developed to separate the products of liver microsomal testosterone metabolism. The microsomal mixture undergoes liquid–liquid extraction and pre-concentration, and then electrophoretic analysis takes less than 25 min including capillary conditioning steps. The development of the complex background electrolyte (Tris–HCl and borate buffers, sodium dodecyl sulfate, β-cyclodextrin and ethanol) necessary for this separation is described. A z-type capillary flow cell is used to obtain adequate detection sensitivity. The proportion in which the metabolites are produced as determined by this method allows assignment of the relative activity of cytochrome *P*-450 enzymes in the microsomes. The technique is useful for comparison of activity in normal and abnormal hepatic microsomes.

Keywords: Testosterone; Steroids

1. Introduction

Cytochrome P-450 isozymes (CYPs) are responsible for most of the NADPH-dependent oxidations of endogenous compounds and xenobiotics. They play a role in activating and detoxifying xenobiotics. One approach to the simultaneous study of the activities of several CYPs is the determination of the regio- and stereoselective hydroxylation of testosterone. Seven pathways of testosterone hydroxylation have been identified and associated with the activities of the following CYPs: CYP1A1, -2A1, -3A1, -2B1, -2C2 (Fig. 1) [1–4].

The testosterone metabolite patterns have been identified from incubations with microsomes or purified enzymes using reversed-phase HPLC and thin-layer chromatographic (TLC) methods for the separation of the metabolites [1,3,4]. The TLC method [1] resolves 13 testosterone metabolites but requires five different solvents. The main advantage of this approach is its high sensitivity due to radiodetection.

Two reversed-phase HPLC methods using gradient elution have been described [4,5]. One is capable of resolving 16 testosterone metabolites [4] and the other only seven (2α, 2β, 6β, 7α, 16α, 16β and androstenedione) [5]. The reported methods are complicated and we have chosen to develop a new approach utilizing capillary electrophoresis (CE).

The method is designed to indicate changes in the proportion of the testosterone metabolites (2α, 2β, 6α, 6β, 7α, 16α, 16β and androstenedione) in microsomal incubates to probe the activities of five

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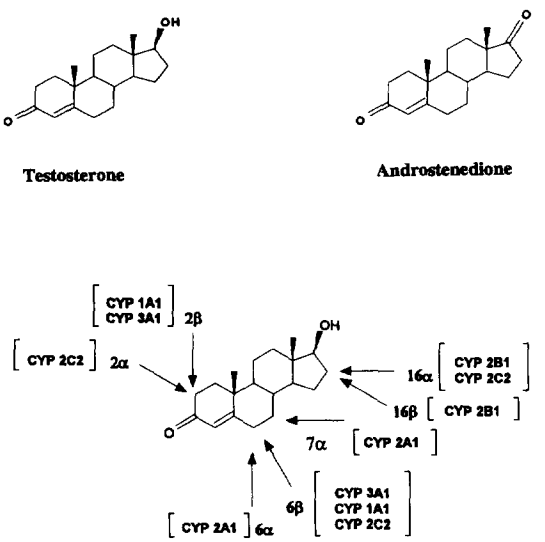

Fig. 1. Structures of testosterone, androstenedione and regioselectivity of testosterone oxidation by CYPs. Abbreviations are used as follows: 6α, 6β, 7α, 16β, 16α, 11α, 11β, 2α, 2β, for the corresponding alcohols.

major CYPs (1A1, 2A1, 3A1, 2B1, 2C2). Our initial application has been in the study of differences in the metabolic profile of testosterone between microsomes from normal and protein-calorie malnourished (PCM) rats [6]. Differences in the relative proportions of each metabolite reveal changes in the activity of each CYP due to PCM. Future work will involve cachectic animals and induction studies. The method has not been validated for the quantitation of each metabolite, since this was not necessary for the intended applications.

2. Materials and methods

2.1. Chemicals

Testosterone (4-androsten-17β-ol-3-one), 6α-hydroxytestosterone (4-androsten-6α,17β-diol-3-one), 6β-hydroxytestosterone (4-androsten-6β,17β-diol-3-one), 7α-hydroxytestosterone (4-androsten-7α,17β-diol-3-one), 16α-hydroxytestosterone (4-androsten-16α,17β-diol-3-one), 16β-hydroxytestosterone (4-androsten-16β,17β-diol-3-one), 2α-hydroxytestosterone (4-androsten-2α,17β-diol-3-one), 2β-hydroxytestosterone (4-androsten-2β,17β-diol-3-one), 11α-hydroxytestosterone (4-androsten-11α,17β-diol-3-one), 11β-hydroxytestosterone (4-androsten-11β,17β-diol-3-one) and androstenedione (4-androsten-3,17-dione) were purchased from Steraloids (Wilton, NH, USA). For the background electrolyte solution (BGE), Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) and β-cyclodextrin were obtained from ICN Biochemicals (Cleveland, OH, USA), sodium borate and boric acid from Anachemia (Montreal, Canada) and sodium dodecyl sulfate (SDS) from Boehringer Mannheim (Indianapolis, IN, USA), all these reagents being analytical grade. Dichloromethane was purchased from Anachemia. Analytical grade magnesium chloride, saccharose, dithiothreitol, EDTA, sodium dihydrogen phosphate and sodium monohydrogen phosphate were purchased from Sigma (St. Louis, MO, USA). Analytical grade potassium dihydrogen phosphate, potassium monohydrogen phosphate and glycerol were from B.D.H. (Toronto, Canada). BSA reagent was purchased from Bio-Rad (Mississauga, Canada).

2.2. Preparation of liver microsomes

Sprague–Dawley (SPF) rats (Charles River, St. Constant, Canada) were sacrificed by exsanguination, under ether anesthesia. Immediately after sacrifice, the livers were weighed and stored at −80°C until the preparation of the microsomes. The livers were thawed, homogenized in a two-fold volume of a

solution composed of sodium phosphate buffer (50 m*M*, pH 7.4), saccharose (250 m*M*), EDTA (10 m*M*) and dithiothreitol (0.1 m*M*) and centrifuged, first at 9000 *g* for 30 min and then at 100 000 *g* for 60 min. All these operations were performed at +4°C. The pellets containing the microsomes were resuspended in the same volume of a potassium phosphate buffer (100 m*M*, pH 8.0) containing EDTA (1 m*M*), dithiothreitol (1 m*M*) and glycerol (20% of the final volume). Aliquots were frozen at −80°C. Protein concentration was determined by the Bradford method [7].

2.3. Incubation and extraction procedures

Liver microsomes were incubated in potassium phosphate buffer (50 m*M*, pH 7.4), magnesium chloride (3 m*M*), EDTA (1 m*M*). The microsome suspension was previously diluted with 250 m*M* saccharose to achieve a final protein concentration of 10 mg/ml. The final protein concentration in the incubation media was 1 mg/ml. Testosterone final concentration was adjusted to 250 μM, according to Sonderfan et al. [4]. Reactions were started by addition of the NADPH-regenerating system (NADPH, 1 m*M*; glucose-6-phosphate, 5 m*M*; glucose-6-phosphate dehydrogenase, 1 unit/ml) and stopped by addition of dichloromethane after 10 min of incubation. The internal standard, 11β-hydroxy-testosterone (4 μl of a 1 mg/ml solution in distilled water–ethanol (60:40, v/v)), was added to each tube. The samples were then extracted by adding 5 ml of dichloromethane [4]. After vortex-mixing for 2 min and centrifuging (2000 *g*, 10 min), the aqueous phase was discarded and the organic phase evaporated under reduced pressure using a Speed Vac SVC 100 system (Savant Instruments, Farmingdale, NY, USA). Residues were reconstituted with 5 μl of ethanol and 20 μl of BGE.

2.4. Capillary electrophoresis conditions

An Applied Biosystems CE system model 270A-HT (ABI Division of Perkin Elmer, Foster City, CA, USA) equipped with a z-cell capillary for enhanced UV detection sensitivity was used for the analysis of testosterone and its metabolites. The capillary was a 72 cm (50 cm to detector) × 75 μm I.D. fused-silica column (LC Packings International, San Francisco, CA, USA). Before each run, preparation of the capillary consisted of a 2-min flush with 0.1 *M* sodium hydroxide and then a 3-min flush with the BGE. The sample was injected by applying a 17 kPa vacuum for 0.5 s. The separation potential was 28 kV, giving a running current of 58 μA. The running buffer was prepared daily, as follows: solutions of Tris (50 m*M*, pH 8.0) and borate buffer (50 m*M*, pH 8.9) were mixed (95:5, v/v). The borate buffer pH was adjusted by adding a 50-m*M* solution of boric acid to the 50 m*M* solution of sodium borate. The pH 8.0 Tris solution was obtained by adding a 50 m*M* HCl solution to a 50 m*M* Tris solution. To this mixture, SDS and β-cyclodextrin were added to give final concentrations of 60 m*M* and 15 m*M*, respectively. This solution was then mixed with ethanol in a ratio of 85:15 (v/v). Detection was performed at λ=240 nm.

2.5. Stock solutions

Stock solutions of testosterone metabolites were prepared in ethanol–distilled water (40:60, v/v). A stock solution of testosterone (0.1 *M*) was prepared in methanol. All solutions were stored at +4°C.

3. Results and discussion

The CE method described in this manuscript allowed the resolution of testosterone and its metabolites. A typical separation is presented in Fig. 2. The order of migration of the compounds was determined by separately injecting a standard solution of each metabolite.

The background electrolyte (BGE) is a complex mixture, which is necessary to resolve all the components. Originally the method was developed using a 50-μm capillary and a relatively high-conductivity pH 7 phosphate electrolyte with an SDS additive to form a micellar pseudophase. Experiments were also performed using a borate buffer and SDS in the BGE, but the change to borate resulted in an almost complete loss of resolution and a considerable reduction in interactions of the analytes with the micellar phase. It was also found that addition of a small proportion of borate to the phosphate-based

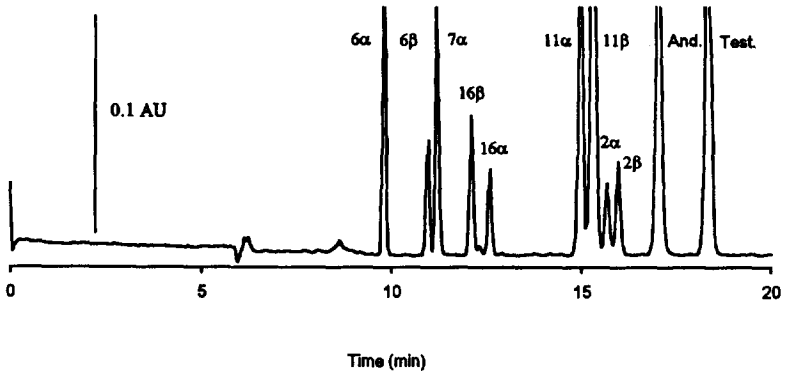

Fig. 2. Electropherogram of a standard mixture of testosterone (Test), 6α, 6β, 7α, 16β, 16α, 2α, 2β-hydroxytestosterone and androstenedione (And). Electrophoresis conditions as in text.

BGE resulted in an improvement in the analyte peak shapes. It seems likely that some degree of complexation between the borate and analytes occurs; formation of a negatively-charged complex would reduce interactions with the SDS micelles, explaining the loss of retention and resolution in the borate buffer system, and would also reduce interactions with the negatively-charged fused-silica walls which could lead to peak tailing. Inadequate limits of detection using a 50-μm capillary necessitated a change to a 75-μm z-cell capillary. A phosphate buffer had too high a conductivity to use in this capillary, necessitating a change to a lower conductivity Tris–HCl buffer. It was also found that, using this buffer, addition of a small amount of borate aided in reducing peak tailing. SDS is necessary to resolve the uncharged compounds of this sample mixture; however with SDS alone, 16α and 16β, and 2α and 2β comigrate. Addition of ethanol to reduce interactions between the analytes and micelles affected primarily the less hydrophobic components and resulted in resolution of 16α and 16β. Further increases in ethanol from the proportion used here lead to loss of resolution of 7α and 6β, while not affecting 2α and 2β. No better results were obtained using other modifiers such as urea which affect primarily the relative hydrophobicity between the aqueous phase and the micellar pseudophase. We therefore investigated additives which may have specific interactions with the analytes. β-Cyclodextrin was found to have little effect on the testosterone metabolites, except for 2α and 2β; addition of β-cyclodextrin allowed resolution of these components.

The highest voltage compatible with baseline resolution of all compounds (28 kV) was used to minimize the separation time. Using the optimized buffer, all the metabolites were baseline resolved. The intra-day coefficient of variation in migration time for each peak was typically ≈0.5% ($n = 8$), allowing easy identification of each component. However, inter-day migration time and effective mobility reproducibility was poor (R.S.D.≈10%, standard samples analyzed on 14 days over a period of 1 month). Nevertheless, the distinctive internal standard peak in each incubation allowed identification of the other peaks, by calculating their relative migration times (RMTs). The inter-day coefficients of variation for the RMT were 3.6% or less for all compounds for the same data set described above (intra-day R.S.D.s in RMT are similar to the actual migration time R.S.D.s, i.e. ≈0.5%). The considerably poor inter-day migration time reproducibility probably reflects the result of minor variations in the buffer, which was made up daily.

The limit of detection (LOD) for testosterone was 0.4 μM ($S/N = 3$). This was approximately ten times better than with a standard capillary (no z-cell). Although the LOD with an ordinary capillary might be adequate for analysis of incubates made with normal microsomes, the PCM microsomes were less active and use of the z-cell became necessary. Quantitation of each analyte is not necessary for

determining which CYPs are affected by different pathological conditions, and thus absolute quantitation was not attempted. Interpretation of the electropherograms consisted of calculating peak areas relative to the IS. These data from study and control groups were compared to reveal differences in the metabolic profiles. Eleven electropherograms made using the test mixture (Fig. 2) over a period of several weeks at concentrations similar to those found in extracts from microsomal incubates revealed an inter-day R.S.D. in relative peak-area measurements of less than 10%, except for the 2α metabolite (13%). In this case the higher R.S.D. probably results from its relatively low concentration in the mixture and from relatively poor resolution from the large 11β peak (which is not present in the actual microsomal incubates).

The absence of interfering compounds from the incubation media was routinely checked with blank incubations being performed in each set of experiments. An electropherogram obtained from an incubation of a mixture of microsomes from normal and from PCM rat livers, without testosterone, is presented in Fig. 3A. A typical electropherogram, obtained from incubation of testosterone with normal rat liver microsomes is presented in Fig. 3B. In comparing these two electropherograms, note that there is a shift in electroosmotic flow, therefore the migration times of the peaks in these electropherograms cannot be directly compared. Instead, as is widely recognized in CE [8], one has to calculate the effective mobilities for these peaks (often such calculations are included in software packages intended for CE data collection, or can be handled externally using a simple spreadsheet). By performing such calculations one can assign the two peaks at 9–10 min in Fig. 3A to those present at around 7.5 min in Fig. 3B. The background peak at around 12.5 min comigrates with the large 6β peak in Fig. 3B. The background peaks at around 15.5 min and 20 min appear in Fig. 3B as a small doublet at around 11 min, and the small peak between 11β and 2α, respectively.

The between-run washing procedure was used to maintain a stable electroosmotic flow and remove any adsorbed components. Occasionally, the electroosmotic flow was observed to decrease considerably and peak broadening was noted. In such cases, the

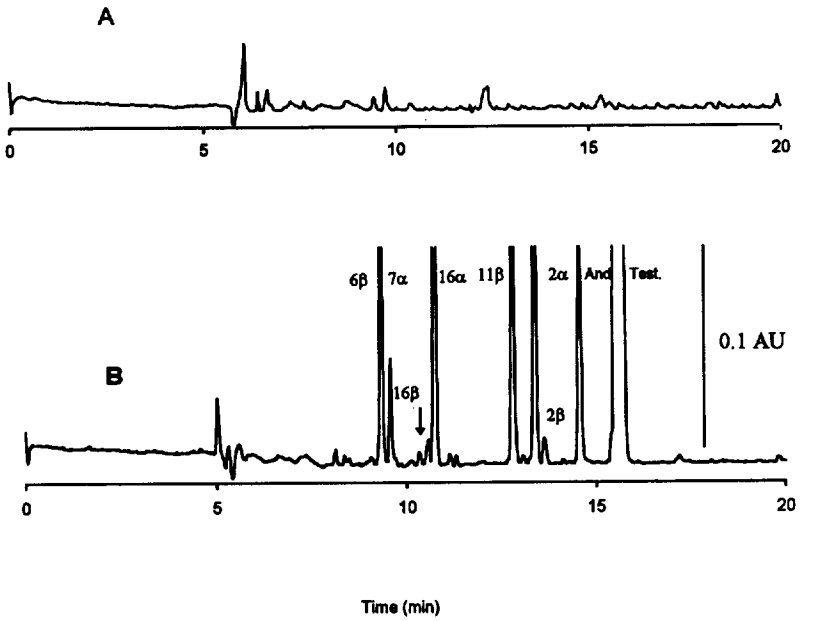

Fig. 3. Electropherograms of: (A) control and PCM microsome mixture incubated without testosterone, (B) testosterone and its hydroxylated products catalyzed by hepatic microsomes from control rats (electrophoresis conditions as in text).

capillary was washed with 1 *M* nitric acid, water and the BGE (15 min each). This was an effective cleaning procedure, but was not incorporated into between-run washes because of the time necessary for capillary re-equilibration [9].

Using the optimized CE conditions, the 11α- and 11β-metabolites were the least well resolved. However, Sonderfan et al. [4] have demonstrated that the incubation conditions used in this study do not produce these compounds. Thus, 11β- was selected as the internal standard.

In comparison with the previously reported HPLC methods, this method is fast and simple. While the extraction procedure used in this study is the same as the one previously described [4], direct sampling from the microsomal incubate, avoiding the extraction step, can be used. In such a case, the SDS present in the running buffer inhibits matrix–wall interactions [10]. However, this method was used to compare the metabolic activity of microsomes prepared from control and protein malnourished rats and with some experimental groups, the amounts of metabolites produced in the incubations were too low to use direct sampling. Therefore, in order to keep same protocol for control and PCM samples, the same liquid–liquid extraction was used for all the samples. The results of this study will be reported elsewhere [6].

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References

[1] D.J. Waxman, A. Ko and C. Walsh, J. Biol. Chem., 258 (1983) 11937.
[2] D.J. Waxman, Biochem. Pharmacol., 37 (1988) 71.
[3] A.W. Wood, D.E. Ryan, P.E. Thomas and W. Levin, J. Biol. Chem., 258 (1983) 8839.
[4] A.J. Sonderfan, M.P. Arlotto, D.R. Dutton and S.K. McMillen, Arch. Biochem. Biophys., 255 (1987) 27.
[5] T. van der Hoeven, Biochem. Biophys. Res. Commun., 100 (1981) 1285.
[6] C. Fernandez, H. Parenteau, D.K. Lloyd and I.W. Wainer, Pharmacol. Commun., submitted for publication.
[7] M.M. Bradford, Anal. Biochem., 72 (1976) 248.
[8] M.T. Ackermans, F.M. Everaerts and J.L. Beckers, J. Chromatogr., 585 (1991) 123.
[9] D.K. Lloyd, in E. Reid, H.M. Hill and I.D. Wilson (Editors), Methodological Surveys in Bioanalysis of Drugs, Volume 23, Royal Society of Chemistry, Cambridge, 1994, pp. 41.
[10] H. Wätzig and D.K. Lloyd, Electrophoresis, 16 (1995) 57.