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Short Communication

Improved sample preparation for the testosterone hydroxylation assay using disposable extraction columns

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ABSTRACT

The preparation of samples for injection into a high-performance liquid chromatograph from assay mixtures for the determination of cytochrome P-450-dependent testosterone hydroxylation has been substantially facilitated. By replacing the multiple cumbersome extraction steps of the conventional method with a single column extraction the time for sample preparation was reduced from hours to minutes. The new procedure also yields better recoveries for most of the testosterone metabolites than the original protocol. The use of extraction columns for sample preparation allows the simultaneous treatment of a large number of samples or even the automation of the whole assay procedure. The modified procedure is a straightforward, easy-to-perform method that should greatly facilitate the implementation of the testosterone hydroxylation assay for sharply discriminating between many individual cytochrome P-450 species in routine enzyme diagnostics.

INTRODUCTION

Oxidative biotransformation is a common fate of many therapeutically used drugs, resulting in the alteration (usually reduction) of the biological activity of these compounds [1]. In addition, a variety of substances are known to be activated to carcinogenic species by this mechanism [2]. The marked inter-individual difference in the capability of performing specific steps in the biotransformation cascade, as observed in humans, may cause a unique susceptibility of each individual to a specific drug treatment and to the carcinogenic effects of respective xenobiotics.

Cytochrome P-450-dependent monooxygenases, a large superfamily of isozymes [3], are regarded as the major enzymes involved in the oxidative metabolism of foreign compounds. The expression pattern for cytochrome P-450 isozymes should thus be an important factor for the above-discussed individual predisposition. The assessment of the cytochrome P-450 expression pattern in livers of laboratory animals has been greatly improved with the introduction of the testosterone hydroxylation assay by Van der Hoeven [4]. Since many of the P-450s studied to date are capable of the hydroxylation of testosterone with regio- and stereospecificities which sharply discriminate individual isozymes [5,6], this *in vitro*

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assay offers the possibility of the simultaneous determination of several different isozymes by the generation of a metabolic fingerprint. Recently, the metabolic profiles of eleven P-450 isozymes of human origin have been assessed, including the site-specific hydroxylation of testosterone [7].

While the amount of information that may be extracted from the result of a testosterone hydroxylation analysis is intriguing, the inconvenience of the method itself is a major obstacle as the sample preparation is complicated and time-consuming. In this paper we describe a simplification of the sample preparation that makes it easy to handle and reduces the time spent for manipulation by an order of magnitude. The new protocol involves a single liquid–liquid extraction step performed on disposable extraction columns, a procedure that has earlier been applied successfully to the quantitative extraction of steroids and related compounds from organic matrices [8,9].

EXPERIMENTAL

Reagents

Testosterone was purchased from Sigma (Deisenhofen, Germany). Standards for the different hydroxylated testosterone metabolites were obtained from Paesel (Frankfurt, Germany) and from the Steroid Reference Collection (London, UK). NADP, glucose-6-phosphate and yeast glucose-6-phosphate reductase, grade II, were from Boehringer Mannheim (Mannheim, Germany). High-performance liquid chromatography (HPLC)-grade methanol was purchased from Promochem (Wesel, Germany). ChemElut extraction columns from Analytichem were obtained through ICT (Frankfurt, Germany).

Instrumentation

Chromatography was performed on a Waters HPLC system including WISP 712 autosampler, two Model 510 pumps directed by an automated gradient controller, a Model 484 tunable absorbance detector and a Model 745 data module for recording and processing of the data. Separation was carried out on a Supelcosil LC-18-DB (5 μm) column (250 mm × 4.6 mm I.D.). (Supelco, Bellefonte, PA, USA).

Incubation conditions

The reaction mixture for the determination of the cytochrome P-450-dependent metabolism of testosterone was composed of an NADPH-regenerating system (10 m*M* glucose-6-phosphate, 0.8 m*M* NADP, 4 U/ml glucose-6-phosphate dehydrogenase) in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (20 m*M*, pH 7.4) containing magnesium chloride (6.7 m*M*), potassium chloride (40 m*M*) and rat liver microsomes (0.5 mg protein/ml). The reaction was started after 2 min of preincubation at 37°C in a shaking water-bath by the addition of testosterone from a methanolic stock solution (80 m*M*) to yield a final concentration of 1.33 m*M*, and incubation was continued for a further 10 min. Thereafter, termination of the enzymatic reaction was accomplished as described for the two different extraction procedures in the following subsections.

Sample preparation by conventional liquid–liquid extraction

Sample preparation according to the method of Van der Hoeven [4] was carried out as follows. The enzymatic reaction was terminated by the addition of 5 ml of dichloromethane to 2 ml of the incubation mixture. A 100-μl volume of a corticosterone solution (methanolic stock solution, 13 m*M*) was added as the internal standard. The resulting mixture was vortex-mixed for 1 min and subsequently centrifuged for 10 min at 1200 *g* in a bench-top centrifuge. The lower, organic phase was recovered and the remaining aqueous phase was extracted with an additional 2 ml of dichloromethane in the same way. The combined organic fractions were extracted once with 2 ml of 20 m*M* sodium hydroxide solution and three times with 2 ml of water, taking care not to disturb the white interphase while removing the upper, aqueous layer. After the final extraction, the organic phase was recovered by passing it over a tissue-paper placed in a Pasteur pipette to re-

move any solid matter. The tissue paper was rinsed with 0.5 ml of dichloromethane for the complete recovery of the extracted steroids and the combined organic eluates were evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was completely dissolved in 333 μl of methanol and an aliquot was taken for subsequent HPLC analysis.

Sample preparation by column extraction

The enzymatic reaction was stopped by the addition of 200 μl of 60 m*M* sodium hydroxide solution, and 30 μl of 13 m*M* methanolic corticosterone solution, to 600 μl of the incubation mixture. The sample was then applied on a Chem-Elut column (maximum holding capacity 1 ml). After 5 min, extraction was started by passing 4 ml of dichloromethane through the column. This step was repeated once and the combined eluates were subsequently evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was completely dissolved in 100 μl of methanol and an aliquot was taken for subsequent HPLC analysis.

HPLC analysis

Separation of the metabolites was performed by injecting 10-μl aliquots of the different preparations into the system. The starting conditions were 65% of eluent A [water–tetrahydrofuran (93:7)] and 35% of eluent B [methanol–tetrahydrofuran (93:7)] at a flow-rate of 0.8 ml/min. For the elution of metabolites a linear gradient was established leading from the starting conditions to 35% of eluent A and 65% of eluent B within 32 min. For the elution of testosterone and subsequent regeneration of the column the concentration of eluent B was raised to 100% within 2 min and was kept constant for another 16 min. Thereafter, the starting conditions were re-established and equilibration of the column was allowed for 15 min prior to the application of the next sample. Elution of the metabolites was monitored at 240 nm. Automatic integration of peak areas was performed by the connected data module. Peaks were normalized using the internal standard as the reference.

RESULTS AND DISCUSSION

The recovery of the internal standard using extraction columns was determined to be 90 ± 3% compared with 80 ± 5% obtained with the conventional [4] extraction procedure. Interestingly, the recovery for many of the testosterone metabolites appeared to be improved by the new method even after normalization to the internal standard, as shown in Table I. The improved recovery as reflected by the increased yield of internal standard may be explained by the unavoidable loss of material during the multiple extraction steps of the standard procedure that are circumvented by the new method. The additional effect on the yield of some of the testosterone metabolites may indicate a higher efficiency of the column extraction for the recovery of metabolites that are more intimately associated with biomatrix molecules such as membrane proteins. It is not dependent on insufficient termination of the enzymatic reaction, as has been demonstrated by a number of additional experiments such as prolonged storage on ice after termination of the enzymatic reaction prior to extraction (data not shown). In the new procedure the recovery was remarkably constant (88–92%) for the individual metabolites when added for calibration after termination of the enzymatic reaction.

We have been using the column extraction procedure extensively in recent months. After several hundred separations on a single HPLC column, we have not encountered any disadvantage of this method in comparison with the standard procedure. The speed and simplicity of the sample preparation by column extraction make the formerly complicated testosterone hydroxylation analysis ideally suited for routine enzyme diagnostics.

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TABLE I

RECOVERY OF TESTOSTERONE METABOLITES

Generation and analysis of testosterone metabolites formed by rat liver cytochrome P-450-dependent monooxygenases were performed as described under Experimental. The hydroxylation reaction was performed in a bulk mix to ensure equal amounts of metabolites for all samples to be extracted. At the end of the incubation procedure the pool was subdivided into two sets of four samples, each, which were then processed by the old [4] and new methods for the recovery and analysis of the metabolites as described. Thus, each value represents the mean of four determinations. Metabolites were identified by the use of authentic standards and are given by designation of the position at which the steroid molecule is hydroxylated; AD = androstene-3,17-dione.

Metabolite	Retention time (min)	Specific enzymatic activity (mean ± S.D.) (nmol/mg · min)		Improved yield with new method (% of old method)
		Old method [4]	New method	
2α	26.3	0.403 ± 0.009	0.408 ± 0.017	101
2β	27.4	0.467 ± 0.010	0.475 ± 0.023	102
6α	14.6	0.058 ± 0.003	0.070 ± 0.004	122
6β	18.4	2.012 ± 0.037	2.382 ± 0.074	118
7α	16.9	0.285 ± 0.008	0.380 ± 0.014	133
15β	15.7	0.106 ± 0.004	0.121 ± 0.005	114
16α	20.6	0.733 ± 0.014	0.866 ± 0.026	118
16β	24.2	0.326 ± 0.010	0.349 ± 0.017	107
AD	31.4	0.738 ± 0.021	0.931 ± 0.029	126

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