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Determination of 4-hydroxyandrostenedione in plasma and urine by extractive alkylation and electron-capture gas chromatography

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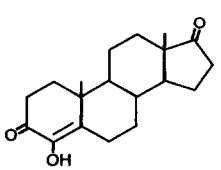
ABSTRACT

A sensitive and specific quantitative assay has been developed for the determination of 4-hydroxyandrostenedione (4-OHA), a potent aromatase inhibitor used in the treatment of estrogen-dependent breast cancer. This steroid has a high first-pass metabolism and is extensively metabolized, mainly by glucuronidation. Plasma levels of unchanged 4-OHA are very low, even after high peroral doses. The analytical method is based on the addition of 17α-ethinylestradiol (internal standard), liquid–liquid extraction from biological material followed by extractive alkylation with pentafluorobenzyl bromide and quantitation by gas chromatography. The method has been validated for sensitivity, accuracy and precision and was found to be suitable for application to pharmacokinetic and bioavailability studies of peroral formulations of 4-OHA.

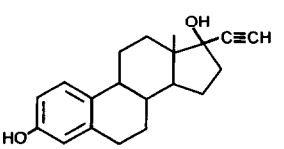
INTRODUCTION

The steroid compound 4-hydroxyandrost-4-ene-3,17-dione (4-OHA, Fig. 1) [1], a potent *in vitro* aromatase inhibitor [2–4], is rapidly metabolized, predominantly by direct O-glucuronidation [5–7]. To study its pharmacokinetics in animals and humans, a sensitive assay was developed.

A previously reported method for the determination of androst-4-ene-3,16-dione, based on the formation to the 3,16-dimethoxime [8], was applied. How-



4-hydroxyandrostenedione



17α-ethinylestradiol
(Internal standard)

Fig. 1. Structures of 4-hydroxyandrostenedione and of the internal standard, 17α-ethinylestradiol.

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ever, the sensitivity of the reaction product was not sufficient, and the peak shape of the derivative was not satisfactory. Another method involves the determination of androst-4-ene-3,16-dione by high-performance liquid chromatography (HPLC) without derivatization [9]. A gas chromatographic–mass spectrometric (GC–MS) method for the determination of free and conjugated 4-OHA in urine was reported [5]. This assay was based on extraction with ethyl acetate, derivatization with octafluorotoluene and tetrabutylammonium hydrogensulphate before determination by GC–MS. It was used for the determination of concentrations in the range 0.2–1.3 μmol/ml in urine. Another method is based on extraction with diethyl ether followed by a chromatographic step with Lipidex 5000, and quantitation by radioimmunoassay (RIA) [3,4,10]. The lower limit of detection of this assay is claimed to be *ca.* 1 pmol/ml of plasma. The most sensitive and specific method published is based on solid-phase extraction, derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA) and quantification by GC–MS [11].

Extractive alkylation is a well established analytical procedure and has been well reviewed [12,13]. Direct extractive alkylation of 4-OHA in the biological material resulted in unsatisfactory chromatographic separation from endogenous constituents. The procedure described here is based on a liquid–liquid extraction followed by extractive alkylation. The extractive alkylation of 4-OHA was carried out with pentafluorobenzyl bromide (PFBBr) as an alkylating agent and with tetrabutylammonium as the counter-ion. The PFB derivative of 4-OHA was found to be stable and to possess excellent chromatographic properties, such as thermal stability and high sensitivity in the electron-capture detector. The synthetic steroid 17α-ethinylestradiol (Fig. 1) was used as an internal standard. For the determination of the sum of free and conjugated 4-OHA an enzymic hydrolysis step is included in the assay.

EXPERIMENTAL

Reagents and chemicals

Stock solutions of 4-hydroxyandrostenedione ($C_{19}H_{26}O_3$; Ciba-Geigy, Basel, Switzerland) and 17α-ethinylestradiol ($C_{20}H_{24}O_2$; Sigma, Deisenhofen, Germany) were made in methanol (Laboratory grade, Ciba-Geigy).

PFBBr was obtained from Fluka (Buchs, Switzerland) and tetrabutylammonium hydrogensulphate from Labkemi (Gothenburg, Sweden). Laboratory-grade 0.1 *M* sodium hydroxide was used. The β-glucuronidase–arylsulphatase solution was obtained from Boehringer (Mannheim, Germany).

Phosphate buffer (pH 7.00) was prepared by dissolving 0.041 mol of disodium hydrogenphosphate and 0.028 mol of potassium dihydrogenphosphate in water to make 1 l of solution. Acetate buffer (pH 5.12) was prepared by dissolving 1.795 mol of sodium acetate and 0.825 mol of acetic acid in water to make 1 l of solution. Diethyl ether, dichloromethane, methanol and toluene (Fluka) were distilled over a 100-cm Vigreux column.

Extraction

The extraction of 4-OHA and the internal standard 17α-ethinylestradiol was optimized by spiking 1 ml of human plasma samples with 1 nmol of 4-OHA, adjusting the samples to various pH values using 0.1 and 1.0 *M* NaOH or HCl. The samples were then extracted and processed as described in *Analytical procedures*. The extractability was calculated by comparing the peak heights of these samples with the peak heights of injected purified derivatives. The optimal pH for the extraction was between 3 and 7. To verify this finding, the optimization procedure was repeated using tritiated 4-OHA and liquid scintillation counting. The results compared well with the standard technique (Fig. 2).

The overall yield for the entire analytical procedure was found to be *ca.* 75%. The influence of the amount of plasma on the recovery of 4-OHA was tested by analysis of plasma samples of 0.1, 0.25, 0.5 and 1.0 ml, all spiked with 0.331 nmol of 4-OHA. The amounts found, after analysis, ranged from 98.5 to 101.5% of the theoretical amount and showed no influence of the amount of biological material used. The extractability from human, dog and rat plasma was found to be the same.

Extractive alkylation

The extractive alkylation step was optimized by varying the reaction time between 5 and 240 min. It was found that the yield did not increase any more after 60 min. To verify the structures of the derivatives, 50 mg of 4-OHA and the internal standard were derivatized by direct reaction with PFBBr (0.5 ml) and potassium carbonate (0.5 g) in 50 ml of acetone by refluxing overnight. Both derivatives were verified by MS to be mono-PFB derivatives.

Stability

The total amount of 4-OHA in plasma or urine samples was stable for at least

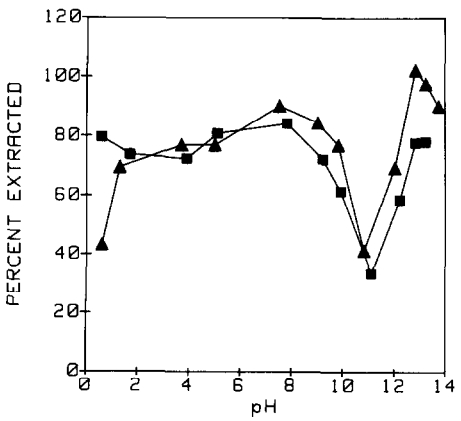

Fig. 2. Dependence on pH of the extraction of 4-OHA (■) and [^{14}C]-4-OHA (▲) from plasma.

four weeks, if stored at −20°C in the dark. However, conjugates may be partially hydrolysed upon repeated thawing and refreezing of the samples. Stock solutions of 4-OHA and the internal standard in methanol were stable for at least four weeks, if stored at 4°C in the dark. The tetrabutylammonium hydrogensulphate solution was also stable for *ca.* four weeks at 4°C. The solution of PFBBr in dichloromethane was stable for *ca.* one week at 4°C.

Analytical procedures

Free compound. A 0.2-ml aliquot of the methanolic solution of the internal standard (0.53 nmol of 17α-ethinylestradiol) was pipetted into a tube and evaporated to dryness under a stream of dry nitrogen at 40°C. To the dry residue were added 0.1–2.0 ml of plasma, 2 ml of phosphate buffer (pH 7.0) and 4.0 ml of diethyl ether–dichloromethane (4:1, v/v). The mixture was shaken for 10 min on a mechanical horizontal rotary shaker (Infors, Basel, Switzerland) at 150 rpm. After brief centrifugation, the organic phase was removed and evaporated to dryness under a stream of nitrogen at 40°C. To the dry residue were added 1 ml of 0.1 *M* NaOH, 0.1 ml of 0.1 *M* tetrabutylammonium hydrogensulphate (in 0.1 *M* NaOH) and 2 ml of dichloromethane containing 0.5% PFBBr. The mixture was shaken for 60 min at 80 rpm. After brief centrifugation, the aqueous phase was removed by aspiration, and the remaining organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at 40°C. The dry residue was redissolved in 2 ml of toluene and 2-μl aliquots were injected into the gas chromatograph.

Free and conjugated compounds. For the determination of the sum of free and conjugated 4-OHA, 1 ml of plasma or urine, 1 ml of acetate buffer (pH 5.12) and 20 μl of β-glucuronidase–arylsulphatase solution were placed in a 10-ml volumetric flask and incubated at 37°C in a water-bath under agitation, for 15 h. After cooling, the mixtures were made up to 10 ml with distilled water. Aliquots of this solution were then analysed as described. The enzymic hydrolysis of the glucuronide of 4-OHA was optimized using synthetic 4-OHA glucuronide. The recovery of free 4-OHA, using the above procedure, was better than 95%.

Gas chromatography. A Pye Model 304 chromatograph, equipped with a ^{63}Ni electron-capture detector, was used. The column was a 1.5 m × 2 mm I.D. Pyrex glass column packed with 3% OV-225 on Supelcoport, 80–100 mesh. The columns were conditioned by repeated injection (Pye Autojector) of calibration samples until the peak-height ratio remained unchanged from one injection to the next. The carrier gas was nitrogen, at a flow-rate of 40 ml/min. The temperatures were: column oven, 275°C; injector, 250°C; detector 350°C. Under these conditions, the retention times were about 2 and 3 min for the derivatives of the internal standard and 4-OHA, respectively. The lifetime of the columns is relatively short, *i.e. ca.* three weeks during constant use. Chromatograms of plasma and urine extracts from a patient are shown in Fig. 3. Chromatograms of extracts from dog and rat plasma were not different from those from human plasma.

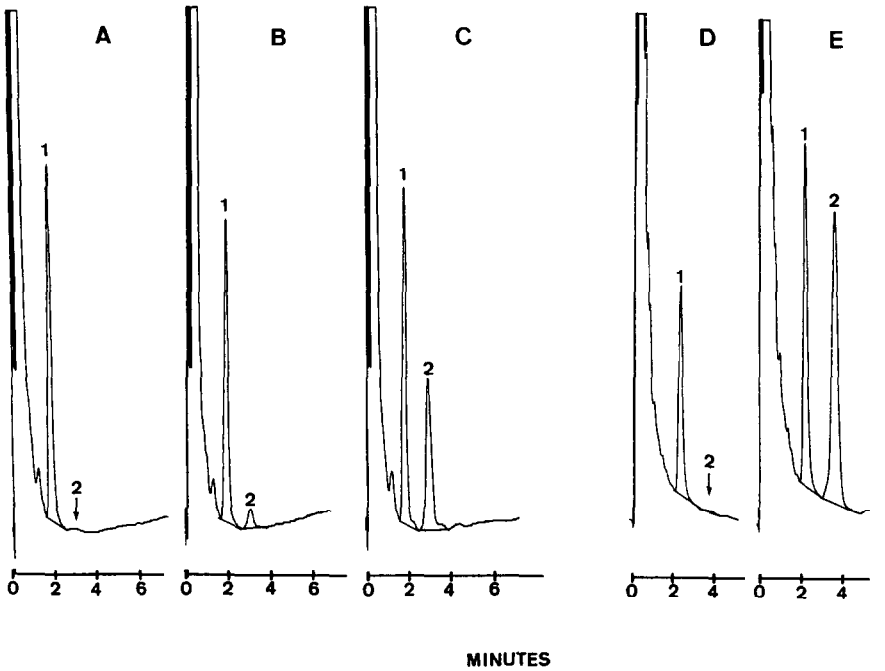

Fig. 3. Chromatograms of extracts of (A) 2 ml of blank plasma, (B) 2 ml of a plasma sample collected from a patient 10 min after treatment with a single peroral dose of 500 mg of 4-OHA, (C) 2 ml of a plasma sample collected 20 min after the dose, (D) 0.2 ml of a blank urine sample and (E) 0.2 ml of a urine sample collected from a patient 0–4 h after treatment with a single peroral dose of 500 mg of 4-OHA. Peaks: 1 = PFB derivative of 17α-ethinylestradiol (0.53 nmol/sample); 2 = PFB derivative of 4-OHA.

RESULTS AND DISCUSSION

Calibration curves

Calibration graphs for plasma and urine determinations were prepared as follows. Blank plasma and urine samples were spiked with 4-OHA (0–0.331 nmol/ml). The samples were then processed as described. The peak height of the 4-OHA derivative was divided by that of the internal standard derivative, and the ratio was plotted against initial 4-OHA concentrations. The quadratic least-squares regression analysis was calculated from the equation $y = a+bx+cx^2$. The parameters of the regression are given in Table I.

Recoveries, precision and limit of quantitation

To validate the method, blank plasma samples ($n = 40$) were spiked with 4-OHA in amounts between 0.020 and 0.866 nmol per ml plasma. The samples were analysed and evaluated by linear least-squares regression ($y = a+bx$). The analysis parameters were as follows: intercept, −0.0052; slope, 1.0169; estimated

TABLE I

PARAMETERS OF THE QUADRATIC LEAST-SQUARES REGRESSION ANALYSIS ($y = a+bx+cx^2$) BETWEEN PEAK-HEIGHT RATIOS AND INITIAL 4-OHA CONCENTRATIONS IN PLASMA AND IN URINE.

Parameter	Plasma	Urine
Correlation coefficient (r)	0.9997	0.9999
Intercept (a)	0.0011	−0.0007
Slope (b)	3.0510	3.1378
Estimated standard deviation (S_y)	0.0098	0.0040

standard deviation (S_y), 0.0153; correlation coefficient (r), 0.9978. The limit of quantitation was 0.05 nmol per sample (15 ng), and the limit of detection was 0.02 nmol per sample (6 ng). In the 0.05–0.843 nmol range, the method validation samples deviated between −13.8% and +14.6% (mean 5.3%).

The method validation in urine samples in the range 0.056–0.470 nmol per ml urine ($n = 25$) gave the following parameters: intercept, 0.0102; slope, 0.9742; S_y, 0.0075; r, 0.9985. In the given range, the found values deviated between −4.8 and +12.5% (mean 4.2%) from the given values.

Blank samples of the plasma and urine materials tested (human, dog and rat; males and females) showed no interfering peaks.

Applicability of the method

In humans (patients) the peak plasma concentrations were in the range 0.17–0.20 nmol/ml after a peroral dose of 250 mg of 4-OHA. The same dose given

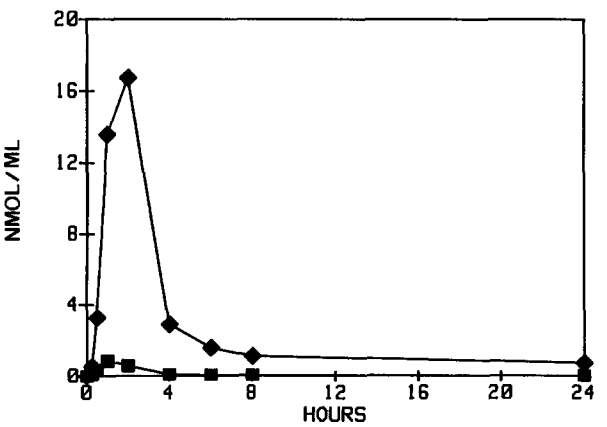

Fig. 4. Concentrations of 4-OHA before (■) and after (◆) enzymic hydrolysis in plasma of a male beagle dog treated with a single peroral dose of 600 mg/kg 4-OHA in a capsule.

intramuscularly produced peak plasma concentrations of *ca.* 0.03 nmol/ml unchanged 4-OHA [14].

Therefore, this GC method is applicable to plasma samples after peroral application and to urine samples (free and conjugated 4-OHA) after both peroral and intramuscular applications. For plasma samples after intramuscular applications, either a radioimmunoassay [3,4,10] or the GC–MS method published recently [11] should be used. In addition, the GC method is suitable for the analysis of plasma samples from toxicokinetic studies in animals.

Application

A male beagle dog was treated with a single peroral dose of 600 mg/kg 4-OHA in a capsule. Plasma concentrations of 4-OHA were measured before and after hydrolysis. The plasma profiles (Fig. 4) show that the major part of the 4-OHA in plasma was present in the form of conjugates. Concentrations of unchanged 4-OHA were low, the peak plasma concentration being 0.88 nmol/ml.

CONCLUSION

Because 4-OHA is extensively metabolized in animals and humans, the plasma levels of unchanged drug are low and a highly sensitive analytical method is required. The assay described is suitable for the monitoring of unchanged 4-OHA, as well as the sum of free and conjugated 4-OHA, in plasma and in urine during pharmacokinetic or bioavailability studies with peroral formulations in animals or humans.

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