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Normal-phase high-performance liquid chromatographic determination of epristeride, a prostatic steroid 5α-reductase enzyme inhibitor, in human plasma

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ABSTRACT

An highly sensitive and selective high-performance liquid chromatographic method was developed for the determination of epristeride [17 β-(N-*tert.*-butyl carboxamido)-androst-3,5-diene-3-carboxylic acid, SK&F 105657], a potent inhibitor of the prostatic steroid 5α-reductase enzyme, in human plasma samples. Epristeride is currently in development for the treatment of benign prostatic hyperplasia. The analytical method involves isolation of epristeride and the internal standard [17 β-(N,N-diisopropyl carboxamido) estra-1,3,5 (10)-triene-3-carboxylic acid, SK&F 105419] from plasma by solid-phase extraction prior to chromatographic separation on an aminopropyl silica column, using hexane–methylene chloride–2-propanol–acetic acid as the mobile phase, with subsequent ultraviolet absorption detection. The absolute recovery of epristeride from plasma was 90.2 ± 2.96. The limit of quantification for epristeride was 2.5 ng/ml. Linear response was observed for concentrations of epristeride ranging from 1 to 500 ng/ml plasma. The assay was sufficiently sensitive, accurate and precise to support pharmacokinetic studies in human subjects.

INTRODUCTION

Epristeride (17 β-(N-*tert.*-butylcarboxamido)-androst-3,5-diene-3-carboxylic acid, SK&F 105657, Fig. 1) is a potent and selective inhibitor of the prostatic steroid 5α-reductase enzyme, which converts testosterone to dihydrotestosterone (DHT) [1–3]. This steroid analogue is currently under development for the treatment of benign prostatic hyperplasia (BPH).

This report describes a sensitive and specific HPLC method for the determination of epristeride in human plasma samples. The approach involves isolation of the steroid analogue from plasma by solid-phase extraction followed by quantitative normal-phase chromatographic analysis with ultraviolet absorbance (UV) detection.

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MATERIALS AND METHODS

Chemicals

Epristeride (free acid, SK&F 105657, Fig. 1) and the internal standard (17 β-(N,N-diisopropyl carboxamido) estra-1,3,5(10)-triene-3-carboxylic acid, SK&F 105419, free acid, I.S.), were supplied by Drug Substances and Products, SmithKline Beecham Pharmaceuticals (Swedeland, PA, USA). Glacial acetic acid was obtained from Mallinckrodt (Paris, KY, USA). Monobasic potassium phosphate, dibasic potassium phosphate, HPLC-grade methanol, hexane, 2-propanol and methylene chloride were obtained from J. T. Baker (Phillipsburg, NJ, USA). Octadecylsilica (C_{18}) solid-phase extraction cartridges (100 mg, 1 ml) and the Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, USA).

Standard solutions and reagents

The stock standard solutions of epristeride and

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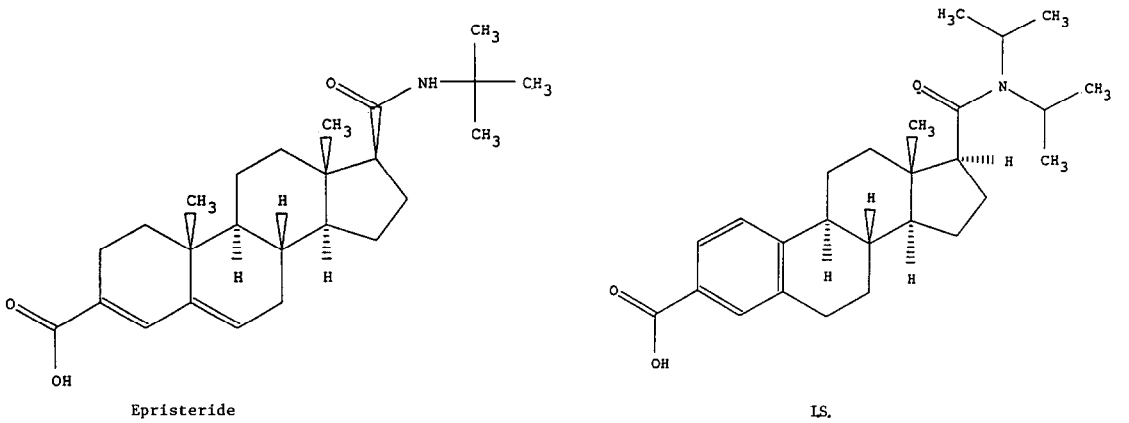

Fig. 1. Structures of episteride and I.S.

the internal standard were prepared by dissolving 10 mg of the free acid in methanol in a 10-ml volumetric flask to give a final solution concentration of 1 mg/ml. Appropriate dilutions of the stock solution of episteride were made with 50% aqueous methanol to generate a series of working standard solutions (100, 10, 1 and 0.1 μg/ml). All stock and working standard solutions were stable for 4 months when stored at 4°C. The stock solution of I.S. was diluted 1:50 with 50% aqueous methanol solution to give a solution concentration of 20 μg/ml. The solution was stable for 2 months when stored at 4°C.

Calibration

A set of plasma calibration standards (concentrations of episteride; 0, 1, 2, 5, 10, 20, 50, 100, 200, 500 ng/ml) was analyzed with every determination of episteride in plasma samples of unknown concentration, by adding appropriate volumes of the working standards of episteride to 1.0 ml of heparinized plasma. The standard curve was established by weighted linear least-squares regression [weighting factor = $1/y$; due to the large difference between low and high concentrations of the analyte, the peak-height ratios (y) were weighted in order to get better estimates of analyte (x) concentrations] of the measured peak-height ratios of episteride to internal standard *versus* the concentration of episteride added to the plasma. Episteride concentrations in unknown plasma samples were calculated from the following regression equation:

$$\text{Concentration of episteride } (x) = (y-a)/b$$

where, b = slope of the regression line, a = y-intercept of regression line and y = peak-height ratio of episteride to internal standard. The linear regression analysis was performed with a FIT FUNTION program of RS/1 software (version 4.3.1, BBN Research Systems, Cambridge, MA, USA). In FIT FUNCTION, the fitting is done in a series of iterations, in which the parameters are systematically adjusted by the Marquardt–Levenberg method until a least-squares solution is reached. The FIT FUNCTION program, besides generating The Equation Of The Best Fit, also compute Goodness-Of-Fit Statistics and generate ANOVA table calculating correlation coefficient (r^2) and standard deviation of the regression and also calculates the 95% confidence intervals for the standard curve.

Mobile phase

Hexane–methylene chloride–2-propanol–glacial acetic acid (410:50:40:1, v/v) is used as mobile phase.

Extraction of episteride from plasma

The C_{18} extraction column was conditioned by successive washings with 1 ml of methanol and 1 ml of water. An aliquot of plasma (1 ml) containing 50 μl of 50% aqueous methanol (contains standards when preparing standard curve), 50 μl of internal standard solution (20 μg/ml, I.S.) and 500 μl of 0.5 *M* phosphate buffer, pH 8.0, were mixed in a 75 ×

12 mm borosilicate tube. The sample was then poured onto the C_{18} column and vacuum was applied. The column was washed successively with 3 ml water, 1 ml of 20% (v/v) aqueous methanol and 1 ml of hexane. The column was then eluted with 1 ml of mobile phase eluent and the eluate was collected into a 75 × 12 mm borosilicate tube. The eluate was evaporated under a gentle stream of nitrogen at 40°C and the residue was reconstituted in 200 μl of mobile phase and transferred to an autosampler vial. 10–75 μl were injected into the HPLC system for analysis.

High-performance liquid chromatography

The isocratic HPLC system consisted of a Beckman pump (Model 116, Beckman, Palo Alto, CA, USA) and an ultraviolet absorbance detector (Model 783, ABI, Ramsey, NJ, USA). Chromatographic separations were carried out on a 22 cm × 2.1 mm I.D. amino-propyl silica column (Pierce, Rockford, IL, USA) connected in-line to a 3 cm × 2.1 mm I.D. amino-propyl silica guard column. The column was maintained at room temperature and the mobile phase eluent, hexane–methylene chloride–2-propanol–glacial acetic acid (409:50:40:1, v/v), was pumped at a flow-rate of 300 μl/min. The mobile phase was filtered through a 0.2-μm nylon-66 filter and degassed before use. UV detection was accomplished at 274 nm. Samples were injected using an HPLC autosampler (WISP model 710B, Waters, Milford, MA, USA). The chromatographic data were collected with an automated laboratory system (Nelson, Cupertino, CA, USA).

Validation procedures

Four pools of plasma precision samples containing 2.5, 25, 250 and 450 ng/ml of epristeride were prepared by adding appropriate volumes of standard solutions to drug-free heparinized plasma. These plasma samples were stored at −20°C until analysis was performed. Five replicate samples from each pool were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. From the data obtained, intra-day precision (determined as the mean of the daily relative standard derivations, R.S.D.s), inter-day precision (determined as the R.S.D. of the daily means) and mean accuracy were calculated.

RESULTS AND DISCUSSION

The assay described here for the analysis of epristeride involves solid-phase extraction of the steroid from plasma as a preliminary isolation step, followed by quantitative HPLC analysis with subsequent UV absorbance detection. The high extinction coefficient of the molecule (exceeding 20 000 at 274 nm) and the use of HPLC columns of reduced internal diameter (2 mm) provided enhanced sensitivity for epristeride in developing highly sensitive analytical methodology for this molecule. The specificity of the method was also attributable to reversed-phase solid-phase extraction of the steroid from plasma samples followed by normal-phase chromatographic analysis of the plasma extract on a amino-propyl silica column, using hexane–methylene chloride–2-propanol–acetic acid as the mobile phase. Use of four component mobile phase is necessary to resolve the analyte and internal standard peaks from the endogenous plasma peaks and to obtain sharper peaks.

Recovery and stability

The recovery of epristeride and the internal standard from plasma was estimated with five determinations by comparing the peak height obtained with processed samples to that obtained by direct injection of an amount of standard equivalent to 100% recovery. At 25 ng/ml, a mean plasma recovery of 90.2 ± 2.96% was obtained for epristeride. In addition, epristeride and internal standard were found to be stable in the final extract at room temperature for at least 48 h.

Sensitivity, selectivity and linearity

By utilizing a 2.1 mm I.D. HPLC column, the on-column limit of detection of epristeride (signal-to-noise ratio 3) was 100 pg. Under the conditions used in this assay, the lowest concentration of epristeride that could be determined quantitatively in 1 ml of plasma samples was 2.5 ng. Calibration curves obtained were linear over the range of 1–500 ng/ml of epristeride. In this range, no interference either from endogenous substances or from the known metabolites of epristeride were observed. Weighted ($1/y$) linear regression analysis of calibration curves provided the equation $y = 0.03635\,x - 0.000307$ and a correlation coefficient greater than

TABLE I

ACCURACY AND PRECISION DATA FOR EPRISTERIDE IN HUMAN PLASMA

Parameter	Concentrations in plasma (ng/ml)			
	2.5	25	250	450
R.S.D. (%)				
Day 1	6.0	1.8	1.7	3.3
Day 2	4.8	6.3	3.4	5.3
Day 3	8.2	1.0	1.1	3.9
Error (%)[a]				
Day 1	−14.0	−8.1	−0.2	−3.0
Day 2	−18.8	−7.5	−2.4	−1.9
Day 3	−11.2	−14.9	−6.0	−5.8
Inter-day R.S.D.[b]	4.5	4.4	3.0	2.1
Intra-day R.S.D.[c]	6.3	3.0	2.0	4.2
Mean accuracy (%)	85.3	89.5	97.2	96.4

[a] (Calculated concentration − actual concentration)/actual concentration × 100.
[b] Coefficients of variation of daily means.
[c] Mean of the daily R.S.D.s.

0.999. The calibration curves were highly reproducible and the precision, as measured by the R.S.D.s at each of the spiked concentrations, was within 17% across the calibration range. The accuracy, evaluated by the average concentration back-calculated from the composite standard curve, was within 10% of the seeded value at each concentration.

Accuracy and precision

Table I summarizes the results obtained from a three-day validation study in which five replicate-seeded standards at four concentrations, 2.5, 25, 250 and 450 ng/ml, were analyzed each day by this methodology. The mean accuracy of the assay at these concentrations ranged from 85.3 to 97.2%, whereas the intra-day precision, indicated by the mean of the daily R.S.D.s, varied from 2.0 to 6.3%. The reproducibility of the assay was high with inter-day precision, indicated by the R.S.D.s of the daily means, ranging from 2.1 to 4.4%. Similar accuracy and precision results were obtained when the assay

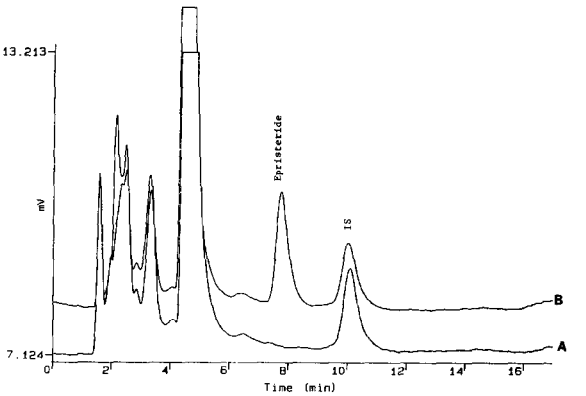

Fig. 2. Chromatograms of plasma extracts from blank human plasma with (A) I.S. and (B) plasma sample spiked with 20 ng/ml of epristeride.

was revalidated with smaller volume of plasma (200 μl), but with a proportional drop in sensitivity.

Application of the procedure to plasma samples

The quantitative HPLC methodology described here provided for selective and sensitive detection of epristeride in human plasma samples. A typical chromatogram of a plasma extract obtained from drug-free plasma and a plasma sample spiked with 20 ng/ml of epristeride is shown in Fig. 2. The chromatography was highly reproducible and provided a retention time for epristeride and the internal standard of 7.9 and 10.2 min, respectively. To date the method has been used successfully in the analysis of biological samples from clinical studies. This method was also adopted for monkey, dog, rat and mouse plasma samples utilizing smaller volumes (100 μl) of plasma for quantification of epristeride in various pre-clinical pharmacokinetic studies.

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