Atmospheric Pressure Photoionization Applied to Quantitation of Cyproterone Acetate in Human Plasma

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Abstract

Cyproterone acetate [6-chloro-1β,2β-dihydro-17α-hydroxy-3'H-cyclopropa(1,2)-pregna-1,4,6-triene-3,20-dione acetate] is a powerful antiandrogen used in the treatment of women suffering from disorders associated with androgenization such as hirsutism and acne. A fast, sensitive, and robustness method is developed for the determination and quantitation of cyproterone acetate in human blood plasma by liquid chromatography coupled with tandem mass spectrometry. Cyproterone acetate is extracted from 0.2 mL human plasma by liquid–liquid extraction. The method has a chromatographic run of 4.5 min, using a C_{18} analytical column (100- × 2.1-mm i.d.), and the linear calibration curve over the range is linear from 1 to 500 ng/mL ($r^2 > 0.994$). The between-run precision, based on the relative standard deviation replicate quality controls, is 96.2% (3 ng/mL), 97.5% (120 ng/mL), and 99.1% (400 ng/mL). The between-run accuracy was ± 2.7%, 3.1%, and 4.8% for the previously mentioned concentrations, respectively. The method is employed in a bioequivalence study of two tablet formulations of cyproterone acetate (100 mg).

Introduction

Cyproterone acetate [6-chloro-1β, 2β-dihydro-17α-hydroxy-3'H-cyclopropa[1,2]-pregna-1,4,6-triene-3,20-dione acetate], chemical abstract service registry number 427-51-0 (Figure 1), is a powerful antiandrogen. It can be used alone or in combination with, for example, ethinylestradiol, in the treatment of women suffering from disorders associated with androgenization such as hirsutism and acne (1,2). Equally, in elderly men, it used for the therapy of prostate carcinoma. Cyproterone acetate is poorly absorbed from the gastrointestinal tract and is rapidly metabolized by several pathways including hydroxylations and conjugations, in 15β-hydroxycyproterone (2).

Cyproterone acetate has been measured by several methods such as radioimmunoassays (3–6) and high-performance liquid chromatography (HPLC) (7–11). Recently, a fully-automated method was published using online solid-phase extraction coupled to an HPLC system, and the limit of quantitation (LOQ) of this method was 15 ng/mL (12). Gas chromatography coupled to mass spectrometry (MS) was also used with an LOQ of 10 ng/mL with a total analysis time of the 5 min; however, previous derivatization with trifluoroacetic anhydride was required (13). The liquid chromatography coupled to tandem MS (LC–MS–MS) is a highly sensitivity technique used for drug analysis in plasma samples (14,15). The high specificity of tandem MS allows for the discrimination and quantitation of different drugs from coeluting metabolites or from a biological matrix without the need of an extensive clean-up or long chromatographic separation (or both) (15).

In the year 2000, a new ionization mode to LC–MS systems was introduced: atmospheric pressure photoionization (APPI) (16). In APPI the ionization is initiated by 10 eV photons emitted by a krypton discharge lamp. The initial reaction in APPI is the formation of a radical cation of the dopant by 10 eV photons. For this reaction to occur, the ionization energy of the dopant has to be lower than the energy of the photons (for this reason toluene is normally used as dopant, introduced, and vaporized in the auxiliary gas within the heated nebulizer probe), and finally the dopant radical cations ionize the analytes through charge exchange (16,17). The use of an APPI source is capable of providing enhanced sensitivity in relation to the atmospheric pressure chemical ionization (APCI) source for different substances (16), including natural product compounds (18) and drugs in plasma samples (19–21).

The main objective of this study was to develop a specific, sensitive, and fast LC–MS–MS method using an APPI source for quantitation of cyproterone acetate in human plasma. This

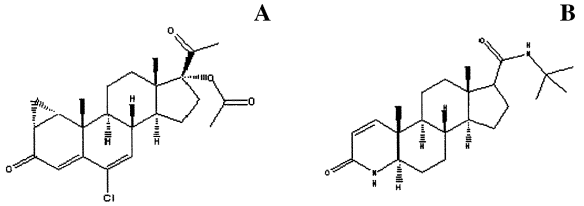

Figure 1. Structures of the cyproterone acetate (A) and finasteride (B).

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method was applied to a bioequivalence study in healthy volunteers.

Experimental

Chemicals and reagents

Cyproterone acetate and finasteride (internal standard, IS) were obtained from Eurofarma (São Paulo, Brazil) and Martinex (Nicosia, Cyprus), respectively. Acetonitrile (HPLC grade), hexane (HPLC grade), and ethyl ether (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ); formic acid (analytical grade) was obtained from Merck (Rio de Janeiro, Brazil). Ultrapure water was obtained from a Gradient Millipore system (São Paulo, Brazil). Blank blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately –70°C until needed.

Calibration standards and quality controls

Stock solutions of cyproterone acetate and finasteride were prepared in acetonitrile–water (50:50) at concentrations of 1 mg/mL. Calibration curves for the cyproterone acetate were prepared in blank human plasma at concentrations of 1, 2, 5, 10, 20, 50, 100, 200, and 500 ng/mL and performed in duplicate in each batch. Quality control (QC) samples were prepared in blank plasma at concentrations of 3, 120, and 400 ng/mL (QCA, QCB, and QCC, respectively).

Sample preparation

Aliquots (0.50 mL) of human plasma were employed for liquid–liquid extraction (LLE) with addition of IS solution (50 µL of the working standard solution at 100 ng/mL). The tubes were vortex mixed for 20 s and allowed to stand at room temperature for 2 min. Four milliliters of diethyl ether–hexane (80:20, v/v) were added and the samples were vortex mixed for 40 s; the upper layer was transferred to clean tubes and the solvent evaporated under N_2 (40°C). The dry residue was redissolved with 200 µL of a mobile phase of acetonitrile–water (80:20 v/v, with 10mM of formic acid). The samples were transferred into glass microvials, capped, and placed in an autosampler.

LC and MS conditions

An HPLC system (LC10AD, Shimadzu, Kyoto, Japan) consisting of a pump and autosampler was used for all analysis. The chromatographic system consisted of a C_{18} Genesis analytical column (100- × 2.1-mm i.d., 4-µm film thickness) (Grace Vydac, Hesperia, CA), and the mobile phase was a mixture of acetonitrile–water (80:20, v/v) at a flow rate of 350 µL/min. The total run time was set for 4.5 min. The column was operated at room temperature with a void time of 0.8 min. The temperature of the autosampler was maintained at 8.0°C and was set up to make a 40-µL sample injection. MS was performed in a Sciex API 3000 triple stage quadrupole MS (Applied Biosystems, Foster City, CA), equipped with an APPI source operating in positive mode (PS+). The source block temperature was set at 300°C and the photoionization capillary voltage at 1.7 kV. Nitrogen was used as the collision gas. For comparison, an electrospray ionization source was also employed and the capillary voltage set at 4.5 kV. The ions monitored in multiple reaction monitoring (MRM), using both sources, were the same (conditions described in Table I). The transitions *m/z* 417.2 → 357.2 and *m/z* 373.1 → 317.4 were used for quantitation of cyproterone acetate and finasteride (IS), respectively. Data were acquired by Analyst software (1.3.1, Applied Biosystems, Cheshire, U.K.) and calibrations curves for the analyte were constructed using the cyproterone acetate and IS peak-area ration via a weighted ($1/x^2$) least-squares linear regression. Unknown sample peak-area ratios were then interpolated from the calibration curve to provide concentrations of cyproterone acetate.

Recovery

Preliminary experiments were conducted to evaluate the recovery with the extraction method described previously. The percentage recovery was calculated as the ratio of the peak area for extracted blank plasma spiked at each standard concentration (3, 120, and 400 ng/mL) relative to peak area of the equivalent blank plasma samples spiked after the extraction.

Stability

Quality control samples prepared to test stability (3, 120, and 400 ng/mL) were subjected to short-term (6 h) room temperature, three freeze-and-thaw cycles, long term (28 days), and 24-h autosampler (8°C) stability tests. Subsequently the cyproterone acetate concentrations were measured in comparison with freshly prepared samples.

Precision and accuracy

The within- and between-run precision was determined as the relative standard deviation (RSD), RSD (%) = 100 (SD/M) and the accuracy as the percentage relative error (RE), RE (%) = (E – T)

Table I. Conditions of MRM in APPI and ESI

Compound	Source	Transition (*m/z*)	IS (V)	Temp. (°C)	Declustering potential (V)	Focusing potential (V)	Collision energy (eV)	Cell exit potential (V)
Cyproterone acetate	APPI	417.2/357.2	1700	300	40	200	25	28
	ESI	417.2/357.3	4500	450	40	170	25	22
Finasteride	APPI	373.1/317.4	1700	300	45	190	37	22
	ESI	373.1/305.4	4500	450	65	170	25	22

(100/T), where M is the mean, SD is the standard deviation of M, E is the experimentally determined concentration, and T is the theoretical concentration.

Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablet formulations of ciproterone (100 mg) in healthy volunteers: ciproterone (test formulation lot no. CIP100LG01, expiration date January 2005 from EMS Indústria Farmacêutica Ltd., Hortolandia, Brazil) and Androcur (standard reference formulation lot no. 14616A, expiration date November, 2006 from Shering do Brasil, Química e Farmacêutica Ltd.).

Fifty-three healthy male volunteers were selected for the study. The study was a single dose, two-way randomized crossover design with a 3-week washout period between the doses. Blood samples were collected before and 0.5, 0.75, 1, 1.15, 1.50, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, 48, 72, 96, 120, and 144 h post-dosing.

The bioequivalence between the two formulations was assessed according to United States Food and Drug Administration (U.S. FDA) methodology (22).

Results and Discussion

The cyproterone acetate and finasteride (IS) formed protonated molecular ions $[M + H]^+$, as the base peaks, in both source types studied [APPI and electrospray ionization (ESI)] (Figures 2 and 3), despite the fact that the ionization processes in these two sources are totally different. In positive ionization with an ESI source, the molecule is protonated or forms adducts (e.g., Na^+ or NH_4^+). This process is assisted by a high potential (3–6 kV). In APPI, the ionization process is dopant assisted (normally toluene is used as dopant, as in the present study); in the positive mode, the ionization is initiated by the photoionization of the toluene and formation of the toluene cation. This cation can ionize the solvent molecule by proton transfer, if the proton affinity (PA) of the solvent molecule is higher than that the deprotonated radical cation. The protonated solvent molecules can donate a proton to the analyte molecule, in case the PA of the analyte is higher than the solvent molecule. Alternatively, the dopant radical cation can ionize the analyte directly by charge exchange if the ionization energy of the analyte is lower than that of the radical cation (16).

Because of the high intensity of the *m/z* 417.2 → 357.2 (cyproterone acetate) and *m/z* 373.1 → 317.4 (finasteride) reactions and no detectable interference in plasma samples, these transition reactions were used in the present method. In ESI analysis, the same ions were used for cyproterone acetate; however, for finasteride, a little variation of abundance was observed of the ions *m/z* 317.4 and *m/z* 305.4. In APPI mode, the ion *m/z* 317.4 is more intense than the *m/z* 305.4; in ESI mode, the ion *m/z* 305.4 is more intense than the *m/z* 317.4.

With these reactions, a specific LC–MS–MS assay to determine cyproterone acetate from human plasma with a validated LOQ of 1.0 ng/mL and run time of less than 4.5 min has been developed. The main criteria for the choice of LOQ value was the presence of

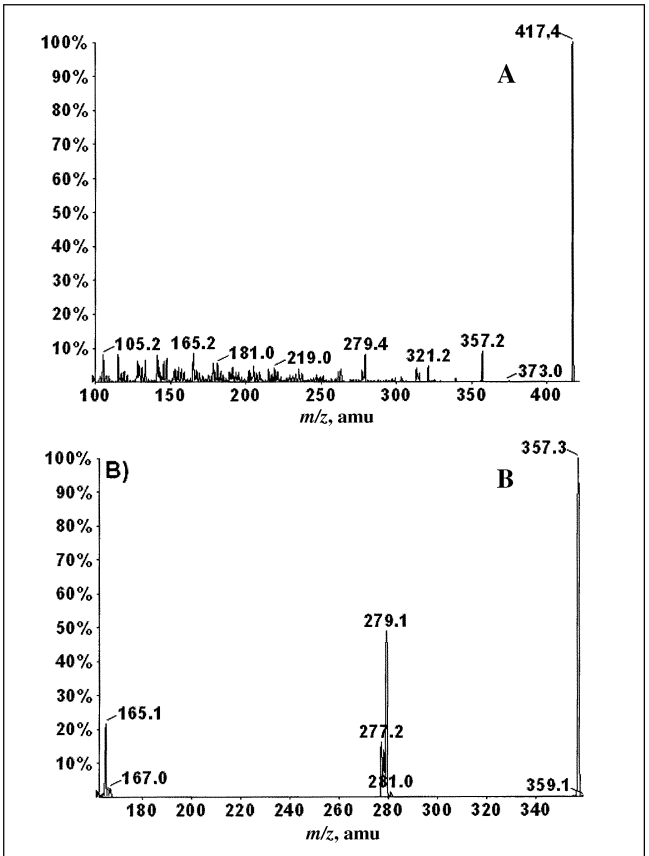

Figure 2. Full-scan mass spectra (Q1) of the cyproterone acetate in APPI mode (A) and their respective product ion spectra (B).

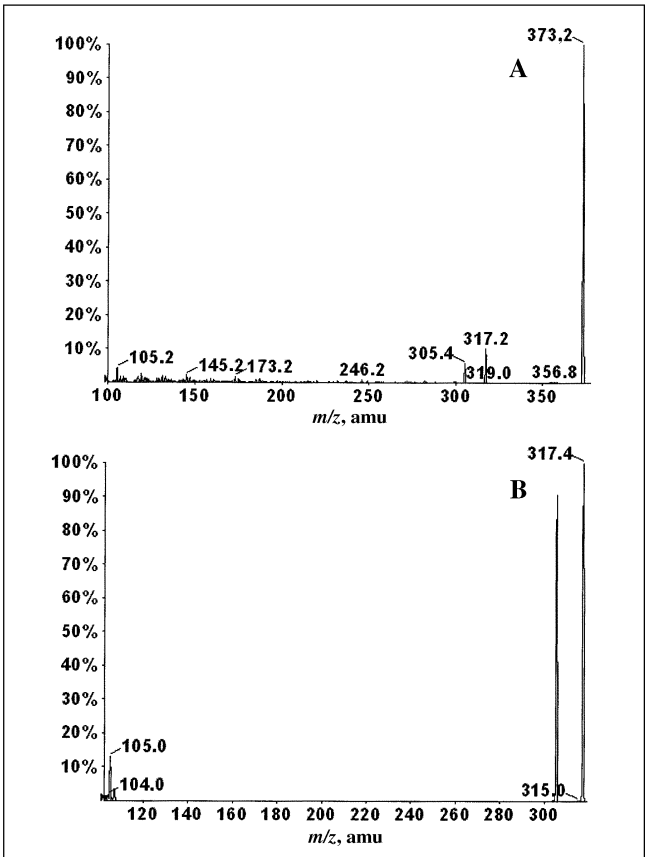

Figure 3. Full-scan mass spectra (Q1) of the finasteride in APPI mode (A) and their respective product ion spectra (B).

no interference in blanks at the retention time of the analyte or a response 7 times greater than any interference in blanks at that retention time. The mass chromatograms of a LOQ sample are shown in Figure 4, in which the retention times of cyproterone acetate and IS were 2.0 and 1.7 min, respectively.

The choice of finasteride as the IS for cyproterone acetate was based on the presence of similar functional groups in both structures and similarity of physical-chemical properties in addition to their similarity concerning molecular weight and chemical behavior.

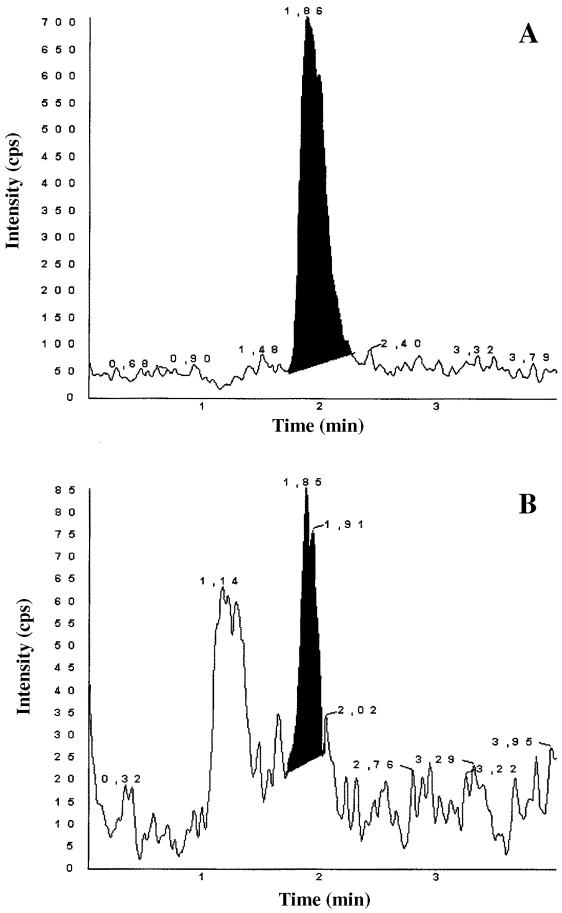

Figure 4. MRM chromatograms of the cyproterone acetate in human plasma at a final concentration of 1 ng/mL: APPI mode (A) and ESI mode (B).

Table II. Validations with the Quality Controls Having the Results of the Accuracy and Precision of the Cyproterone Acetate

		Nominal concentration (ng/mL)			
	Parameter	1.0	3.0	120	400
Intrabatch	Mean found (n = 8) (ng/mL)	0.82	2.80	117	370
	CV (%)	16.9	3.1	5.1	6.4
	Precision (%)	82.0	93.3	97.5	94.8
Interbatch	Mean found (n = 3) (ng/mL)	0.98	2.89	117	396
	CV (%)	14.3	2.7	3.1	4.8
	Accuracy (%)	98.0	96.2	97.5	99.1

Assay performance

The accuracy and precision of the method was assessed by analyzing the QC samples. The method was linear for cyproterone acetate from 1 to 500 ng/mL ($r^2 > 0.9970$) on repeated calibration curves. The optimized method was validated by assessment of recovery, linearity, quantitation limit, precision, and accuracy. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the LOQ, whose values were extended to 20%, as recommended by Shah et al. (23) and Bressole et al. (24) for the analysis of biological samples for pharmacokinetics studies.

The recovery of cyproterone acetate, calculated from the peak area ratios of extracted human plasma previously spiked at final concentrations of 1.0, 120, and 400 ng/mL (n = 15 for each concentration) were 92.4%, 85.6%, and 83.9%, respectively. For the IS (1.0, 120, and 400 ng/mL), the recoveries were 104.4%, 103.8%, and 96.3%, respectively. No matrix effect was observed. Between- and within-run accuracy and precision for the quality controls are summarized in Table II.

The stability tests performed indicated no significant degradation under the conditions previously described, including in the freeze and thaw test, short-term room temperature test, and long-term test (28 days). The human plasma spiked at final concentrations of 3, 120, and 400 ng/mL (n = 5 for each concentration). In the latter case (long term test), a variation of +10.3%, –6.9%, and –2.7%, respectively, were determined relative to freshly spiked samples.

Comparison of the LC–MS–MS sources

During the prevalidation studies, the same MS, APPI, and ESI sources were used, and the results show that the both source systems exhibited good specificity without matrix interferences, however the LC–APPI-MS–MS is a more sensitive system for cyproterone acetate analysis. The MS conditions are shown in Table I. In the LOQ (1 ng/mL), the typical signal noise with the ESI source was 5, and with the APPI source was 81, showing a difference of sensitivity of the APPI in relation to ESI higher than 10 times. The LOQ chromatograms obtained with the APPI and ESI sources are shown in Figure 4. Probably because the presence two nitrogen atoms in the finasteride structure promote an increase in molecular polarity and the sensitivity in ESI source, a signal noise was observed in ESI mode that was six times higher than in APPI mode. These results show that some of the potential advantages of the use APPI, as an ionization approach to increase the detection studies in complex matrixes (e.g., human plasma), are more evident for low polar compounds.

Bioequivalence

The geometric mean and respective 90% confidence interval (Cl) of ciproterone/androcur percent ratios were 100.68% (96.56–104.97%) for the area under the concentration time curve (AUC) of AUC_{last}, 99.02% (94.84–103.38%) for $AUC_{0\text{-}inf}$, and 88.15% (81.68–95.13%) for C_{max}. After the oral administration of the ciproterone tablets to the volunteers, the observed ciproterone peak plasma mean concentration (C_{max}) values are in Figure 5.

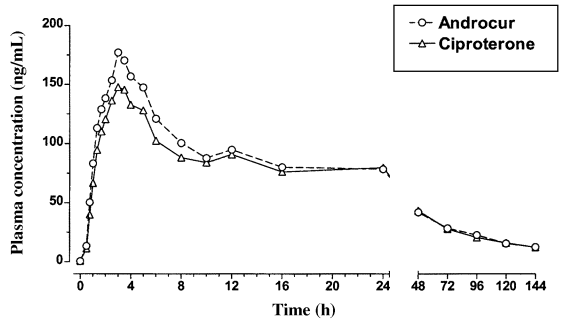

Figure 5. Plasma mean concentration of cyproterone acetate.

In addition, the calculated 90% CI for mean C_{max}, AUC_{last}, and AUC_{inf} ciproterone/androcur individual ratios were within the 80–125% interval defined by the U.S. FDA (22).

Conclusion

An LC–MS–MS method for the quantitation of the cyproterone acetate in human plasma was developed and validated using photospray ionization mode. This ionization mode is five times more sensitive than the ESI mode to cyproterone acetate.

This method offers advantages over those previously reported, in terms of a simple sample extraction; only LLE is needed without clean-up procedures for a faster run time (4.5 min). The LOQ of 1.0 ng/mL is sufficient for the pharmacokinetics studies (the estimate $LOQ/C_{max} < 3\%$). The assay performance results indicate that the method is precise and accurate enough for the routine determination of the cyproterone acetate in human plasma.

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