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Fully automated method for the liquid chromatographic determination of cyproterone acetate in plasma using restricted access material for sample pre-treatment

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Abstract

A new fully automated method for the quantitative analysis of an antiandrogenic substance, cyproterone acetate (CPA), in plasma samples has been developed using on-line solid-phase extraction (SPE) prior to the determination by reversed-phase liquid chromatography (LC). The automated method was based on the use of a precolumn packed with an internal-surface reversed-phase packing material (LiChrospher RP-4 ADS) for sample clean-up coupled to LC analysis on an octadecyl stationary phase using a column-switching system. A 200-μl volume of plasma sample was injected directly on the precolumn packed with restricted access material using a mixture of water–acetonitrile (90:10, v/v) as washing liquid. The analyte was then eluted in the back-flush mode with the LC mobile phase which consisted of a mixture of phosphate buffer, pH 7.0–acetonitrile (54:46, v/v). The elution profiles of CPA and blank plasma samples on the precolumn and the time needed for analyte transfer from the precolumn to the analytical column were determined. Different compositions of washing liquid and mobile phase were tested to reduce the interference of plasma endogenous components. UV detection was achieved at 280 nm. Finally, the developed method was validated using a new approach, namely the application of the accuracy profile based on the interval confidence at 90% of the total measurement error (bias+standard deviation). The limit of quantification of cyproterone acetate in plasma was determined at 15 ng ml^{-1}. The validated method should be applicable to the determination of CPA in patients treated by at least 50 mg day^{-1}.

Keywords: Cyproterone acetate

1. Introduction

Cyproterone acetate (6-chloro-1β,2β-dihydro-17α - hydroxy - 3′H - cyclopropa[1,2] - pregna - 1,4,6-triene-3,20-dione acetate) (CPA) (Fig. 1) has antiandrogenic and some progestogenic and antigonadotropic properties that result in the partial suppression of pituitary gonadotropin, and a decrease in serum testosterone. It is used for the control of libido in severe hypersexuality or sexual deviation in adult males. It is also used in males for the palliative treatment of prostatic carcinoma and may be prescribed jointly with ethinylestradiol [1] in females for the control of severe acne and idiopathic hirsut-

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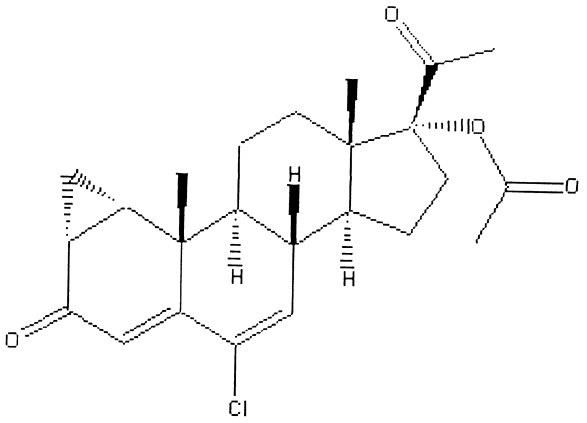

Fig. 1. Structure of cyproterone acetate. Molecular mass=416.95. Molecular formula: 6-chloro-17-hydroxy-1α,2α-methylenepregna-4,6-diene-3,20-dione acetate.

ism. Cyproterone acetate is poorly absorbed from the gastrointestinal tract and is rapidly metabolized by various pathways, including hydroxylations and conjugations, in 15β-hydroxycyproterone. It is slowly excreted in feces and urine [2]. The administered dose depends on the indication. In practice, the usual dosage is 100 mg twice daily [3] for the treatment of prostate carcinoma and 100 mg or less for the treatment of acne and hirsutism [4]. The average concentration of CPA detected in plasma after administration of 100 mg CPA day^{-1} (Androcur®) is about 100 ng ml^{-1}. The ingestion of Diane®-35 (association of 2 mg of CPA and 35 μg of estrogenic compound) leads to a maximal serum level (C_{max}) of 15 ng cyproterone acetate ml^{-1} at 1.6 h [5,6].

Several methods have been developed for the quantitative analysis of CPA in pharmaceutical forms [7,8]. They are based on liquid chromatography (LC). Other LC methods have been also described for the assay of CPA in biological fluids using liquid–liquid extraction [9–13] or column-switching technique [14] for sample clean-up. A limit of quantification (LOQ) of about 50 ng ml^{-1} was usually obtained by applying these bioanalytical methods.

The column-switching technique is an interesting alternative to liquid–liquid or off-line solid-phase extraction for the preparation of biological samples. This automated technique involves the use of two columns connected by a switching valve and is particularly useful when a large number of assays has to be performed. The role of the first column (precolumn) is to retain selectively the analytes. After rotation of the switching valve, the latter are eluted and transferred to the analytical column and individually quantitated [15].

A new concept in LC packing materials for bioanalytical purposes was introduced in 1985 by Pinkerton and coworkers [16,17]. The basic idea was to develop a material that eliminated the risk of denaturing the proteins, but still retained the small molecules like drugs and related compounds. This was accomplished by producing a solid phase that exposed a hydrophilic external surface towards the large biomolecules, and, simultaneously, a rather hydrophobic internal surface towards the small molecules.

Boos and co-workers introduced a new class of restricted access material [18–24] called alkyl-diol silica (ADS) that can be packed in small precolumns used for the sample clean-up of protein-rich samples in column-switching systems. ADS precolumn lifetime corresponds approximately to 200 injections of 500-μl plasma samples [24].

This kind of internal-surface reversed-phase packing material presents two different surfaces: hydrophilic diol groups are bound to the external surface of the silica particles (25 μm) and prevent the adsorption of proteins while the internal surface consists of a hydrophobic C_4, C_8 or C_{18} bonded phase accessible to low-molecular-mass compounds such as drugs or metabolites. The LiChrospher ADS column has a pore diameter of about 6 nm and presents a molecular mass cut-off of 15 000 daltons. This kind of restricted access support packed in small precolumns has been applied successively to the clean-up of biological samples in column-switching systems [25–33].

A novel approach used for the validation of the developed method was based on the mean bias as well as its two-sided 90% confidence limits by using the standard deviation for intermediate precision. The calculated bias for each concentration level was plotted as a function of the concentration in order to obtain an accuracy profile. The acceptance limits were settled at ±20%, as it is usually the case for bioanalytical methods.

The aim of this work was to develop and validate

a fully automated coupled-column LC method for the determination of cyproterone acetate in plasma.

The first part of the study consists of the optimisation of the LC conditions in order to obtain a sufficient retention of the analyte on the stationary phase of the analytical column.

The second part focuses on with the development of the sample preparation procedure and in particular the selection of the appropriate washing liquid and valve-switching time.

Finally, the method developed is pre-validated and validated using the new approach.

2. Experimental

2.1. Chemical and reagents

Cyproterone acetate was purchased from Sicor (Lerma, Mexico). Sodium dihydrogenphosphate dihydrate and sodium hydroxide were of analytical grade from Merck (Darmstadt, Germany). Acetonitrile was of HPLC grade from Merck.

The water used in all experiments was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

The stainless steel analytical column (125×4 mm I.D.) was packed with LiChrospher RP-18 (5 μm) stationary phase from Merck.

The precolumn was a LiChroCart column (25×4 mm I.D.) packed with LiChrospher RP-4 ADS (25 μm), generously donated by Professor K.-S. Boos from Ludwig-Maximilians University of Munich (Munich, Germany).

2.2. Apparatus

A Gilson ASPEC XL combined on-line with an LC system was used. It consisted of an auto-sampling injector, a Model 402 dilutor equipped with a 5-ml syringe and two Rheodyne Model 7010 six-port switching valves (Berkeley, CA, USA).

The chromatographic system was composed of the following units: two Model LC-10AD pumps, a Model SPD-10A UV–Vis detector, a Model CT0-10AC programmable column oven and a Model CBM-10A communication bus module, all from Shimadzu (s'Hertogenbosh, The Netherlands).

A Model Lo-Pulse LP-21 from Alltech (Laarne, Belgium) was used to reduce residual fluctuations in the detector baseline due to the pump.

2.3. Chromatographic conditions

All chromatographic experiments were carried out in the isocratic mode, using a mobile phase of 15 m*M* sodium phosphate buffer, pH 7.0–acetonitrile (54:46, v/v) at a flow-rate of 1 ml min^{-1}.

The liquid used for sample loading and clean-up was water–acetonitrile (90:10, v/v) and was delivered by pump 1 at a flow-rate of 2 ml min^{-1}.

The analytical column was thermostatted at 25 °C in the programmable oven and UV detection was performed at 280 nm.

2.4. Solutions

2.4.1. Solutions for method development

A stock solution containing 1 mg ml^{-1} of cyproterone acetate was prepared in acetonitrile. A 200-times dilution in acetonitrile permitted one to obtain an intermediate solution (5 μg ml^{-1}). All solutions used during the method development were obtained by diluting 10% of this intermediate solution in 90% of water or plasma matrix in order to obtain 500 ng ml^{-1} of CPA.

2.4.2. Solutions used for method pre-validation

A stock solution containing cyproterone acetate was prepared by dissolving 20 mg of bulk powder in 20 ml of acetonitrile. A 1-ml volume of this solution was then diluted with acetonitrile in a 100-ml flask in order to obtain a daughter solution of CPA (10 μg ml^{-1}). This solution was then diluted with acetonitrile in order to obtain a concentration range (0.1, 0.5, 1, 2 and 5 μg ml^{-1}).

These solutions were used to spike plasma samples (2 ml) over the considered range (from 5 to 500 ng ml^{-1}).

Seven concentration levels were selected, one at the supposed LOQ (20 ng ml^{-1}), one below the selected LOQ (5 ng ml^{-1}), one at the maximal concentration studied (500 ng ml^{-1}) and four intermediate concentrations (10, 50, 100 and 200 ng ml^{-1}). In the calibration range three repetitions were made at each concentration level and three series of calibration were performed in pre-validation.

In order to determine the response function, several regression models were investigated: simple linear model, linear model passing through 0, quadratic model and linear model after logarithmic transformation. The selection of the most suitable model was based on the examination of the accuracy profiles established by considering the four regression models. In practice, the concentration of each calibration sample was calculated by considering each regression model. The mean bias and the standard deviation for intermediate precision were calculated for each level of concentration. From all these collected results, the confidence limits at 90% of the total measurement error (bias+standard deviation of intermediate precision) were then calculated for each concentration level.

2.4.3. Solutions used for method validation

The stock and daughter solutions (0.15, 0.2, 0.5, 2 and 5 μg ml^{-1}) were prepared with the same protocol used for the pre-validation step (see Section 2.4.2). These solutions were used to spike plasma samples (2 ml) over the considered range (from 15 to 500 ng ml^{-1}) in order to prepare calibration and validation samples.

For calibration, five concentration levels were selected, one at the estimated limit of quantification (15 ng ml^{-1}), one at the maximal concentration studied (500 ng ml^{-1}) and three intermediate concentrations (20, 50 and 200 ng ml^{-1}). The sequence of injections consisted in three repetitions of the extreme concentrations and one injection for the other levels.

Three series of calibration samples were performed during the validation step.

Five concentration levels (15, 20, 50, 200 and 500 ng ml^{-1}) were selected for the validation samples in order to cover the whole range. In the validation range four repetitions were made at each concentration level for 3 days.

2.4.4. Sample preparation procedure

The plasma sample was first centrifuged at 4500 rpm for 10 min and a 1.8-ml volume of plasma was mixed with 0.2 ml of acetonitrile. The mixture was then centrifuged at 4500 rpm for 10 min and the supernatant was transferred into a vial on the appropriate rack of the autosampler. All the other operations were then executed automatically.

The sample preparation was performed in two steps:

(1) Injection and washing of sample: a 200-μl volume of plasma sample was injected, the column-switching being set according to Fig. 2A. The sample was washed for 10 min with a mixture of water–acetonitrile (90:10, v/v). The flow-rate was 2 ml min^{-1}. Macromolecules such of proteins and other hydrophilic endogenous compounds were eliminated from the precolumn while the analyte was retained. Meanwhile, the analytical column was re-equilibrated with the LC mobile phase.

(2) Elution of the analyte from the precolumn and LC analysis: after 10 min, the valve was switched in order to connect the precolumn with the analytical column. CPA was eluted in the back-flush mode by the LC mobile phase (cf. Fig. 2B). Four minutes later, the switching valve was returned to its initial position and the precolumn was re-equilibrated with the washing liquid. In the meantime, the LC analysis was performed on the analytical column.

3. Results and discussion

3.1. Optimisation of the LC conditions

The optimisation of the LC conditions for the determination of CPA in hydro–alcoholic solutions was carried out by the study of the influence of two organic modifiers (acetonitrile and methanol) on the retention (in terms of retention factor, k') and peak symmetry (in terms of symmetry factor, A_s) of CPA.

The aim of the selection of the organic modifier was to obtain a sufficient retention of the peak corresponding to CPA and to reach as much as possible a good peak symmetry. These two parameters have to be optimised in order to improve the selectivity of the bioanalytical method. The optimisation step was performed by injecting directly solutions of CPA on the analytical column using different mobile phases (cf. Table 1) at a flow-rate of 1 ml min^{-1}. As seen in Table 1, better results with respect to peak symmetry were obtained with acetonitrile.

However, when using an LC mobile phase consist-

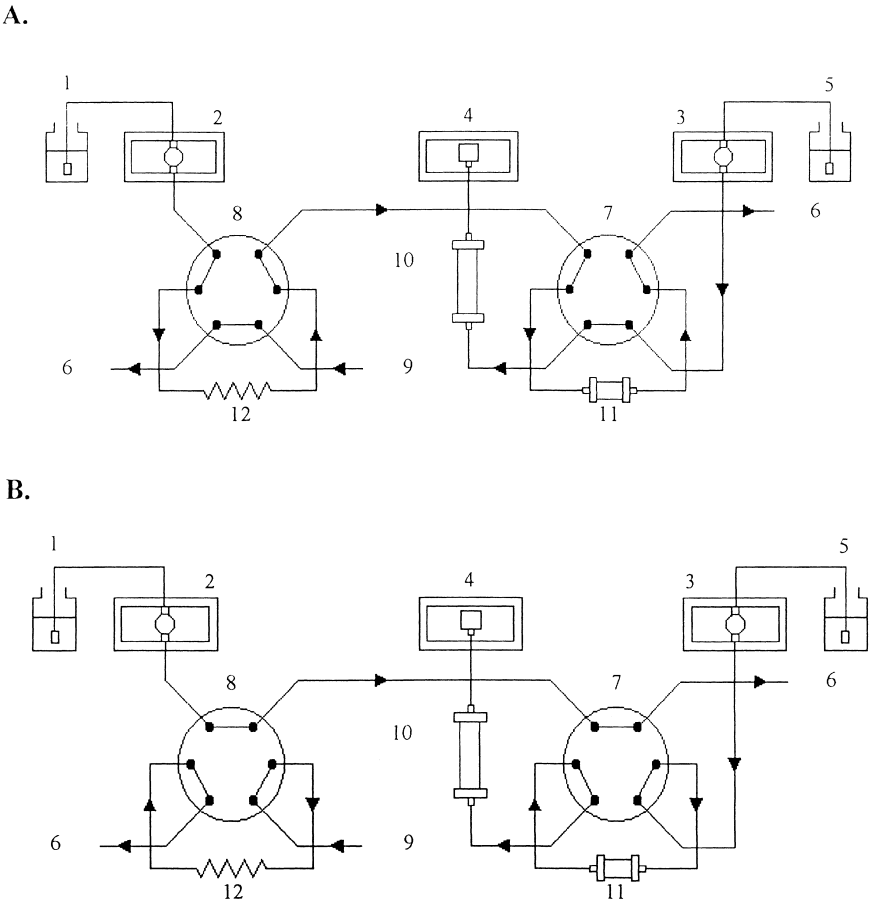

Fig. 2. Schematic representation of a column-switching system. 1=Washing liquid; 2=washing liquid pump 1; 3=liquid chromatographic pump 2; 4=detector; 5=liquid chromatographic mobile phase; 6=waste; 7=switching valve; 8=injection valve; 9=sample loading; 10=analytical column; 11=precolumn; 12=injection loop.

ing of water–acetonitrile (54:46, v/v), an interference from the plasma matrix was observed on the chromatogram at the retention time of the peak corresponding to CPA. Mobile phases containing pH 3.0 and 7.0 buffers were then tested. The interference could be eliminated by selecting an LC mobile phase

Table 1
Effect of organic modifier on the symmetry and on the retention factor of the peak of cyproterone acetate

Composition of the mobile phase	Retention factor (k')	Symmetry factor (A_s)
Water–methanol (50:50, v/v)	15.43	1.32
Water–methanol (65:35, v/v)	4.41	1.31
Water–methanol (70:30, v/v)	2.30	1.37
Water–acetonitrile (50:50,v/v)	4.98	0.99
Water–acetonitrile (54:46, v/v)	4.01	1.00
Water–acetonitrile (65:35, v/v)	1.57	1.20

Column: LiChrospher 100 RP-18 (125×4.0 mm I.D., 5 μm), mobile phase: as mentioned in the table above, flow-rate: 1 ml min^{-1}, UV detection at 280 nm, injection: 200 μl of a hydro–alcoholic solution of cyproterone acetate (100 ng ml^{-1}), temperature: 25 °C.

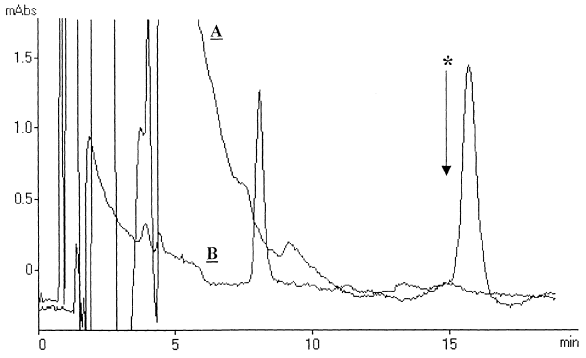

Fig. 3. Influence of the composition of the liquid chromatographic mobile phase on method selectivity. Sample preparation: LiChrospher RP-4 ADS (25×4.0 mm I.D.), washing liquid: water–acetonitrile (90:10, v/v), flow-rate: 2 ml min^{-1}, loading step: 10 min, transfer step: 4 min. Chromatographic analysis: LiChrospher 100 RP-18 (125×4.0 mm I.D., 5 μm), flow-rate: 1 ml min^{-1}, UV detection at 280 nm, injection: 200 μl of blank plasma, temperature: 25 °C, mobile phases: (A) phosphate buffer, pH 3.0–acetonitrile (54:46, v/v); (B) phosphate buffer, pH 7.0–acetonitrile (54:46, v/v). *: Expected retention time of cyproterone acetate.

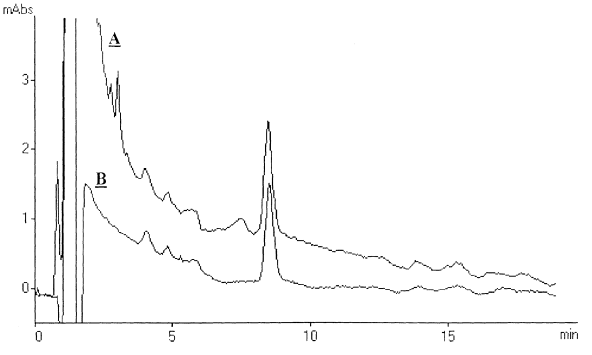

Fig. 4. Chromatograms illustrating the effect of two washing liquids on the elution of plasma matrix by using the column-switching technique. Sample preparation: LiChrospher RP-4 ADS (25×4.0 mm I.D.), flow-rate: 2 ml min^{-1}, loading step: 10 min, transfer step: 4 min, washing liquids: (A) phosphate buffer, pH 3.0–acetonitrile (90:10, v/v); (B) water–acetonitrile (90:10, v/v). Chromatographic analysis: LiChrospher 100 RP-18 (125×4.0 mm I.D., 5 μm), mobile phase: phosphate buffer, pH 7.0–acetonitrile (54:46, v/v), flow-rate: 1 ml min^{-1}, UV detection at 280 nm, injection: 200 μl of blank plasma, temperature: 25 °C.

made of phosphate buffer, pH 7.0–acetonitrile (54:46, v/v) (Fig. 3).

3.2. Development of the sample preparation procedure

In order to develop a method for the determination of CPA in plasma using a column-switching system with a precolumn packed with restricted access material, it was necessary to determine the most appropriate valve-switching times.

3.2.1. Determination of the elution profile of the CPA and selection of the washing liquid

The first step consisted of the determination of the elution profile of the analyte in order to deduce the time from which a beginning of elution of CPA would take place. This time obviously depends on the composition of the washing liquid, its flow-rate and also on the type of solid phase in the precolumn and the dimensions of the latter.

The restricted access material, LiChrospher ADS RP-4, was selected owing to the high hydrophobicity of CPA. In fact, as expected with this kind of sorbent, a sufficient retention of the analyte was observed ($k'>400$).

Two different liquids containing water or phosphate buffer, pH 3.0–acetonitrile (90:10) were tested as washing liquids (Fig. 4). This figure shows that a cleaner chromatogram was obtained with the washing liquid consisting of a mixture of water–acetonitrile (90:10, v/v). Consequently this washing liquid was selected.

3.2.2. Determination of the elution profile of blank plasma

To determine the valve-switching time, it was also necessary to measure the time needed for a complete elution of the proteins present in plasma samples from the precolumn. Such experiments were achieved by directly connecting the precolumn to the UV detector set at 280 nm and by injecting blank plasma samples (200 μl). The flow-rate was set at 2 ml min^{-1}.

A complete elution of proteins was achieved within 5 min with a washing liquid consisting of water–acetonitrile (90:10, v/v). The valve-switching time was therefore set at 10 min. This time was twofold higher than the elution time of the proteins and was still much lower than the time corresponding to the beginning of the elution of CPA (90 min).

3.2.3. Determination of the time needed for analyte transfer

In order to perform the chromatographic separation, the analyte retained on the precolumn must be transferred quantitatively to the analytical column.

The time period for analyte transfer is that required to backflush the analyte from the precolumn to the analytical column. After this time, the switching valve can be returned to its original position. The determination of this time was performed with the detector directly connected to the switching valve.

CPA was eluted rapidly from the RP-4 ADS precolumn due to the much stronger eluting strength of the LC mobile phase (phosphate buffer, pH 7.0–acetonitrile, 54:46, v/v). It took about 3 min to transfer quantitatively CPA from the precolumn to the analytical column.

Finally, a period of time of 4 min was selected for the analyte transfer to the analytical column. Consequently, 14 min after sample injection, the switching valve was returned to its initial position and the RP-4 ADS column was then re-equilibrated with the washing liquid before the next injection. The total analysis time, including sample preparation and chromatographic analysis, was about 25 min.

3.3. Method pre-validation

The validation strategy consisted of two steps: pre-validation and validation.

In order to determine the most appropriate response function, several regression models were investigated: simple linear model, linear model passing through 0, quadratic model and finally linear model after logarithmic transformation. The selection of the most suitable model was based on the examination of the accuracy profiles established by considering the four regression models (Fig. 5A–D). In practice, the concentration of each calibration sample was back-calculated using each regression model. The mean bias and the standard deviation of the intermediate precision were determined for each

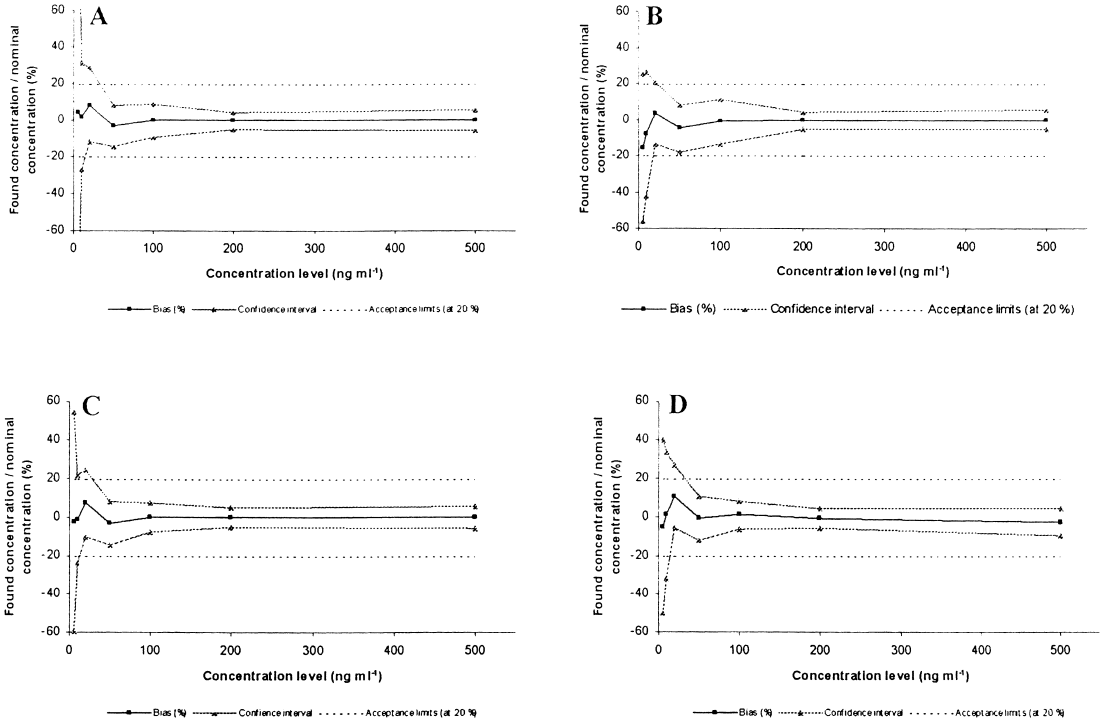

Fig. 5. Accuracy profile obtained in pre-validation by applying several regression models. (A) Linear regression; (B) linear regression passing through 0; (C) quadratic regression; (D) linear regression after logarithmic transformation.

concentration level. From all these results, the confidence limits at 90% of the total error (bias+standard deviation of intermediate precision) were then calculated at each concentration level. The LOQ corresponds to the concentration for which the upper or lower of confidence limits reach +20% or −20%.

Of the four accuracy profiles, the linear model passing through 0 (Fig. 5B) seemed to be the most adequate. Indeed, by applying this model, the calibration range was enlarged and the LOQ was estimated to be close to 15 ng ml^{-1}, the lowest value observed. Moreover, this model is simple to use in routine analysis.

3.4. Method validation

The developed method was validated over a concentration range comprised between 15 and 500 ng ml^{-1} in accordance with the results obtained during the pre-validation step (cf. Fig. 6). At each concentration level, the mean bias as well as the two-sided 90% confidence limits by using the standard deviations for intermediate precision were plotted as a function of the concentration in order to obtain an accuracy profile. The acceptance limits were settled at ±20% for the LOQ and at ±15% for the other concentrations.

Since the confidence limits did not exceed these acceptance limits irrespective of the concentration level, the automated LC procedure developed for the determination of CPA in human plasma can be considered as accurate (in terms of total error) within the concentration range investigated.

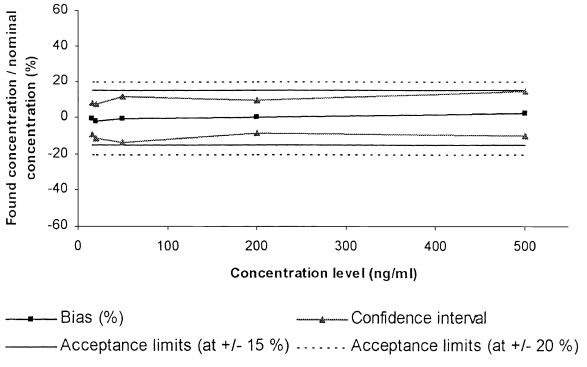

Fig. 6. Accuracy profile obtained during the validation step by applying a linear regression model passing through 0.

Other validation parameters such as selectivity, absolute recovery, precision (repeatability and intermediate precision) were also tested and the LOQ was confirmed [34].

3.4.1. Selectivity

Fig. 7 shows typical chromatograms obtained with a blank plasma sample and a plasma sample spiked with CPA. Under the selected conditions, the mean retention factor for the analyte was equal to 14.0. The absence of interfering endogenous components at the retention time of CPA is clearly demonstrated in this figure.

Moreover, the selectivity of the method was demonstrated by comparing chromatograms of blank plasma samples from different donors with those obtained after analysis of plasma samples from the dosed patient (cf. Fig. 8).

3.4.2. Absolute recovery

The absolute analyte recovery was determined by comparing peak areas obtained from freshly prepared plasma samples and those found by direct injection of aqueous standard solutions at the same concentrations, using the same auto-sampler equipped with the same loop of 200 μl. The absolute recovery for CPA was found to be close to 100% in the investi-

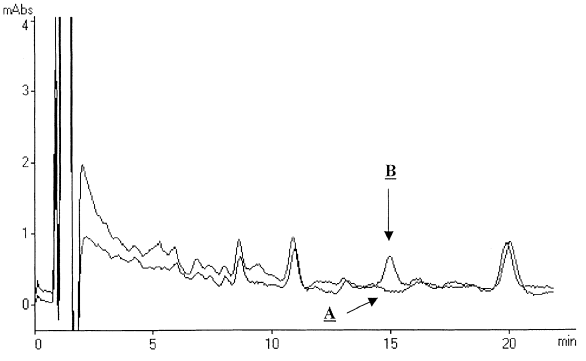

Fig. 7. Method selectivity for the determination of cyproterone acetate in plasma. Samples: blank plasma (A) and spiked plasma with 15 ng ml^{-1} of cyproterone acetate (B). Precolumn: LiChrospher RP-4 ADS (25×4.0 mm I.D.), washing liquid: water–acetonitrile (90:10, v/v), flow-rate: 2 ml min^{-1}, loading step: 10 min, transfer step: 4 min. Column: LiChrospher 100 RP-18 (125×4.0 mm I.D., 5 μm), mobile phase: phosphate buffer, pH 7.0–acetonitrile (54:46, v/v), flow-rate: 1 ml min^{-1}, UV detection at 280 nm, injection: 200 μl of plasma sample, temperature: 25 °C.

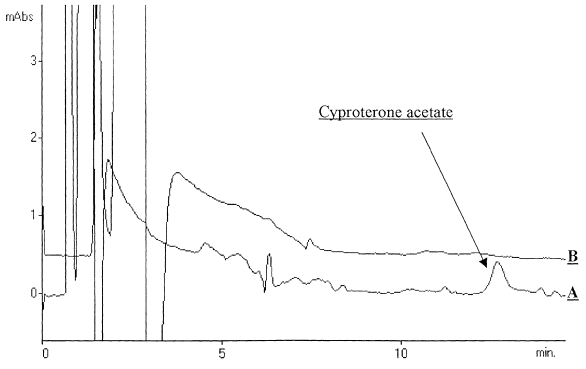

Fig. 8. Method selectivity for the determination of cyproterone acetate in dosed patients. Samples: sample plasma from dosed patient (A) and blank plasma from donor (B). Precolumn: LiChrospher RP-4 ADS (25×4.0 mm I.D.), washing liquid: water–acetonitrile (90:10, v/v), flow-rate: 2 ml min^{-1}, loading step: 10 min, transfer step: 4 min. Column: LiChrospher 100 RP-18 (125×4.0 mm I.D., 5 μm), mobile phase: phosphate buffer, pH 7.0–acetonitrile (54:46, v/v), flow-rate: 1 ml min^{-1}, UV detection at 280 nm, injection: 200 μl of plasma sample, temperature: 25 °C.

gated range of concentration (cf. Table 2). The results obtained show that the absolute recovery is relatively constant over the range considered.

3.4.3. Limit of quantification

The LOQ estimated at 15 ng ml^{-1} during the pre-validation step was confirmed. As can be seen in Fig. 6, the method can be considered as accurate at this concentration level.

3.4.4. Precision

The precision of the automated bioanalytical method was determined by calculating the relative standard deviation (RSD) values for repeatability and intermediate precision at five concentration levels, ranging from 15 to 500 ng ml^{-1}. All results were below 5%, except at the concentration level of 50 ng ml^{-1} for which the RSD for repeatability and intermediate precision were 6.1 and 6.6%, respectively (cf. Table 2).

3.4.5. Precolumn lifetime

In order to maintain an optimal lifetime, it is important to centrifuge the samples, to add an organic modifier to the washing liquid used for the fractionation step and to install a replaceable in-line filter between the sample injector and the precolumn protecting the sieves and tubing from clogging. These different recommendations have been taken into account in the analytical protocol. Under the proposed experimental conditions, the precolumn was shown to work properly for over 400 injections of 200 μl of plasma.

4. Conclusion

A fully automated procedure was developed for the LC determination of cyproterone acetate using restricted access material for sample handling.

The developed method was selective towards endogenous components of plasma in dosed patients and the recovery of the analyte was higher than 90%. Its accuracy was also assessed over a concentration range from 15 to 500 ng ml^{-1}.

These results demonstrate the applicability of the proposed method to determine CPA plasma levels in patients who are treated for prostate carcinoma or other indications with relatively high dosages (i.e., at least 50 mg day^{-1}).

The applicability of this validated method to the analysis of lower dosed samples (see Introduction) involve the choice of an alternative detection tech-

Table 2
Validation of the automated method for the determination of cyproterone acetate in plasma

	Concentration level (ng ml^{-1})				
	15	20	50	200	500
Precision: repeatability/ intermediate precision (RSD, %; n=4 replicates×3 days)	4.6/4.6	3.7/4.5	6.1/6.6	0.7/2.9	2.5/4.8
Absolute recovery (%, mean±SD, n=4)	93.8±3.1	92.0±7.8	93.9±3.3	96.6±3.6	97.9±6.2

nique. The coupling of the sample pre-treatment to mass spectrometry should consequently increase the sensibility of the method. Other parameters such as injection volume should also be modified.

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