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ORIGINAL ARTICLE

# Development of efficient SPE–TLC method and evaluation of biological interactions of contraceptives with progesterone receptors

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Protein bindings

**Abstract** TLC–SPE methodologies were developed to ascertain biological interactions of norethindrone acetate and dydrogesterone contraceptives with plasma progesterone receptor proteins. TLC solvent system for plain and Cu(II) impregnated silica gel plates was *n*-hexane-*n*-butanol (90:10, v/v), which took 20 min to run up to 10.0 cm. The best separation was on Cu(II) impregnated plates due to maximum difference in $R_f$ values and compact spots. The optimized SPE conditions were pH 2.0 and 3.0 of phosphate buffer (50 mM) for norethindrone acetate and dydrogesterone, respectively. The flow rate of plasma and eluting solvent (methanol) through $C_{18}$ cartridge was 0.10 mL/min. The interactions of these contraceptives with progesterone receptor proteins were analysed by TLC–SPE results, which were supported by modelling using PyMOL and Autodoc4 softwares. The dydrogesterone was found to be bound strongly than norethindrone acetate. Attempts have been made to discuss the drugs' interactions at chemo-supramolecular level.


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## 1. Introduction

Nowadays, Asian and African countries are under great pressure due to geometrical growth of population, effecting the economy and ecosystem of the whole world. The control of this alarming problem is essential and an urgent need of today. The population control is being achieved through various contraceptive approaches. During the last four decades, many contraceptive methods have been developed and used; and among them oral dosages are adopted globally (Mansour 2005; Coelingh Bennink et al., 2003). Most commonly used oral contraceptive drugs are based on estrogens and progesterone hormones. Among many, norethindrone acetate

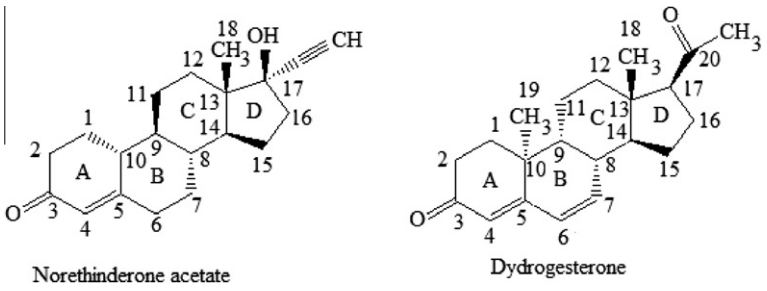


**Figure 1** Chemical structures of norethindrone acetate and dydrogesterone contraceptives.

and dydrogesterone Figure 1 are the most commonly used progestogenic components in hormone replacement therapy (HRT); with about 80% in the market (Coelingh Bennink et al., 2003; Li et al., 2005). These progestogens have been used world widely as oral contraceptive preparations for many years with sales of about billions of US $ per year (Willard et al., 2003). In spite of their crucial role in birth control, these medications result into some side and toxic effects, that require the exploration of the action of mechanism for further improvement in their molecular structures. The interactions of these drugs with protein are the main core of the mechanism of action. Literature indicates that these drugs bind with progesterone receptor proteins (Madauss et al., 2004) but the binding profile and pattern is not well known. These types of studies require the efficient analytical methods and modelling software. A thorough search of literature indicates only few analytical methods for monitoring these drugs. Among them, chromatography is an ideal technique and HPLC (Matlin et al.,1983; Gonzalo-Lumbreras and Izquierdo-Hornillos, 2000; Sundaresan et al., 2006) and TLC (Simard and Lodge, 1970) have been used for this purpose. Of course, HPLC is a more advanced and popular modality, however, thin layer chromatography (TLC) has its unique feature of inexpensiveness, parallel chromatographic profiles of unknowns and standards and ease of operation (Stahl1969; Fried and Sharma, 1991; Fried and Sharma, 1996). Therefore, TLC method development is the real demand for these drugs due to explosion of population in developing countries, where TLC suits well as an ideal technique (inexpensive).The monitoring of analytes in plasma needs sample preparation before loading onto TLC. It has been observed that about 80 percent chromatographers are using solid phase extraction (SPE) as the versatile method of sample preparation for plasma (Ali et al., 2008). It is interesting to observe that no method is available for monitoring these drugs in plasma by using SPE–TLC combination, that we found ideal for third world countries. In view of these facts, attempts have been made to develop inexpensive, fast, selective and reproducible SPE–TLC methods for analyses of the reported contraceptives in human plasma. Based on the results obtained and modelling, efforts were made to explain the interactions of these drugs with progesterone receptor protein for further research. The results of these findings are discussed herein.

## 2. Experimental

### *2.1. Chemicals, reagents and instruments*

Fresh frozen human plasma (Mfg. Licence No. 504) was purchased from Rotary Blood bank, New Delhi India. Methanol, *n*-hexane, *n*-butanol and silica gel G, disodium hydrogen phosphate and *o*-phosphoric acids were purchased from Merck, India. Ferric chloride, sulphuric acid and glacial acetic acids were obtained from Qualigens, India. Standard solutions (0.10 mg $mL^{-1}$) of these drugs were prepared in methanol. Norethindrone acetate and dydrogesterone were detected on TLC plates by developing a new reagent. The reagent was prepared by dissolving 500 mg ferric chloride in a mixture of sulphuric acid (20 mL) and glacial acetic acid (10 mL) followed by water dilution up to 50 mL. Purified water was prepared using a Millipore Milli-Q (Bedford, MA USA) water purification system., Sep-Pak $C_{18}$ 1 mL barrel size cartridges containing 50 mg of sorbent (particle size 55–105 μm and pore size 125 Å) were purchased from Waters USA (Cat.No. WAT054955). pH meter of Control Dynamics (model APX 175 E/C), spectrophotometer of Perkin Elmer (model EZ201), solid phase extraction unit of VARIAN and centrifuge (model C854/49/06) of Remi were used. PyMOL visualization tool and Autodoc4 software were used for modelling purposes of drugs binding with progesterone receptor.

### *2.2. Extraction of drugs from commercial tablets*

Norethindrone acetate and dydrogesterone were extracted from commercially available tablets. Norethindrone acetate was extracted from five regestrone tablets (batch No. R67B05D) of Novartis India Limited, Mahad, (Maharashtra), India. Similarly, dydrogesterone was extracted from five Duphastone formulation (batch No. L7301) of Solvay Pharma India Limited, Mumbai, India. Five tablets of each drug were weighed and crushed to powder separately, respectively. The powdered tablets were extracted with methanol (100 mL), separately, by heating at 80 °C. The drug mixture was centrifuged and supernatant was separated. The residue was extracted two more times with the same amount of methanol separately. All methanol fractions were combined together to get 300 mL. This methanol was evaporated under vacuum on water bath to 15 mL, which was allowed to crystallize in freeze at 10 °C. The mother liquor was decanted and the crystals were washed with a little amount of *n*-hexane. The purity of the drugs was ascertained by melting point, UV and IR spectra.

### *2.3. Preparation of TLC plates*

TLC plates (10 cm × 15 cm × 0.5 mm) were prepared in the laboratory by spreading slurry of silica gel G (50 g) in 100 mL Millipore water. These plates were dried overnight in an oven at 80 °C. For impregnated TLC plates, silica gel slurry was prepared with Millipore water containing 0.10 g each of

$FeSO_4{\cdot}7H_2O$, $CoSO_4{\cdot}7H_2O$, $NiCl_3{\cdot}6H_2O$, $Cu(CH_3COO)_2{\cdot}H_2O$ and $ZnSO_4{\cdot}7H_2O$ in 100 mL Millipore water individually and, respectively. The plates were activated by heating in an oven.

### 2.4. Solid-phase extraction (SPE)

The blank experiments were carried out by mixing 1.0 mL of each drug (1.0 mg $mL^{-1}$) into 5.0 mL Millipore water individually and separately. The spiked sample was vortexed for 2 min, kept for 30 min, mixed with 15.0 mL acetone and centrifuged at 1000 rpm for 5 min to separate the supernatant. The supernatant was evaporated to dryness and the residue was re-dissolved in 10.0 mL phosphate buffer (50 mM, pH 2.0) for norethindrone acetate and pH 3.0 for dydrogesterone. A Sep-Pak Vac $C_{18}$ cartridge (1.0 ml Waters, USA) was pre-conditioned with 2.0 mL methanol followed by 5.0 mL Millipore water. Spiked samples were passed through the cartridge with 0.1 mL flow rate followed by cartridge washing with 2.0 mL Millipore water at the same flow rate. The cartridge was dried by passing hot air and the drugs were eluted by 10.0 mL methanol at 0.1 mL per minute flow rate. The eluted methanol was concentrated under vacuum to 0.5 mL. This solution was used for TLC studies. The same procedure was adopted to analyse these drugs in human plasma.

### 2.5. Thin layer chromatography

TLC conditions were developed for these drugs on plain and impregnated plates. TLC plates were spotted by these drugs at 10.0 ng level with the help of graduated capillaries (10.0 μL) and the chromatograms were developed in TLC chamber pre-equilibrated with solvents for 30 min. The chromatograms were developed up to 10.0 cm for 20 min by using *n*-hexane-*n*-butanol (90:10, v/v) at 20 ± 2 °C. The exhaustive experiments were carried out to optimize the chromatographic conditions. The reported solvent system was optimized by increasing the concentrations of *n*-butanol. After development the plates were dried at 80 °C and the spots were detected by spraying the above cited reagent followed by heating of plates at 80 °C for 1 h. Solvent system was optimized by varying the concentrations of *n*-hexane and *n*-butanol. The analyses of these drugs; after extraction by SPE; were also carried out by TLC using the same developed method. The qualitative determination of these drugs in plasma was ascertained by comparing the $R_f$ values of these with those of standards. For quantitative analyses the spots (10 μL) from five TLC plates were scratched and dissolved in methanol (3.0 mL), which was concentrated to 1.0 mL for determining their concentrations by UV–Vis. Spectrophotometer at 300 nm and 298 nm wavelengths for norethindrone acetate and dydrogesterone, respectively. The calibration curves were linear for both drugs from 0.1 to 0.01 mg/mL concentrations. For validation, all the experiments were carried out five times under the identical experimental conditions and data were validated.

## 3. Result and discussion

### 3.1. Chromatography

$R_f$ values of norethindrone acetate and dydrogesterone were obtained by dividing the distance travelled by these drugs by the solvent front (10.0 cm). The results are given in Table 1, which indicate greater $R_f$ value of norethindrone acetate than dydrogesterone with 0.40 and 0.10 ng as limits of detection (LOD) of these pharmaceuticals, respectively. To optimize the chromatographic condition, various combinations of *n*-hexane and *n*-butanol were tried but the best separation was achieved by using *n*-hexane:*n*-butanol (90:10, v/v) solvent system on plain TLC plates. The variation of mobile phase composition was carried out on plain TLC plates by increasing amount of *n*-butanol. The effect of mobile phase compositions on $R_f$ values is given in Figure 2, which indicates that $R_f$ values increased sharply with 1.0–10.0% of *n*-butanol but became almost constant after this composition. This may be due to rapid increase in polarity from 1.0% to 10.0% *n*-butanol while the further increase of *n*-butanol does not increase polarity; as the concentration of low polarity *n*-hexane becomes negligible. Furthermore, this figure depicts that $R_f$ values of norethindrone acetate are slightly higher than dydrogesterone at all compositions of the mobile phase, which are the characteristic features of these drugs. Furthermore, attempts have been made to improve more separation and, therefore, some TLC experiments on metal ions impregnated plates were also carried out. The selected metal ions for impregnation were Fe(II), Co(II), Ni(II), Cu(II) and Zn(II) of 0.1% concentration each separately. The results on impregnated plates were also recorded in Table 1. A comparison of the separation on plain and impregnated TLC plates from Table 1 clearly shows better results on copper impregnated TLC plates with maximum difference of $R_f$ values and compact spot. On the other hand, the diffused spots were observed on all other metal ions impregnated TLC plates. TLC chromatograms of these two drugs on plain and copper impregnated plates are shown in Figure 3 which indicate better separation of these drugs on copper metal ion impregnated plates. It is very interesting to observe that the color of both the spots was brown on copper impreg-

**Table 1** $R_f$ values and detection limits of norethindrone acetate and dydrogesterone on plain and impregnated silica gel layers with different metal ions.

| Contraceptive drugs | $R_f$ values on plain silica gel layers | $R_f$ values on impregnated silica gel layers | | | | | LOD (ng) |
|---|---|---|---|---|---|---|---|
| | | Fe(II) | Co(II) | Ni(II) | Cu(II) | Zn(II) | |
| Norethindrone acetate | 0.82 | 0.91D | 0.92 | 0.92D | 0.92 | 0.94 | 0.40 |
| Dydrogesterone | 0.73 | 0.68 | 0.74D | 0.84 | 0.82 | 0.87D | 0.10 |

Experimental conditions: D: diffused spots, solvent system: *n*-Hexane:*n*-butanol (90/10,v/v), solvent front: 10.0 cm, developing time: 25 min, room temperature: 20 ± 2 °C, detecting reagent: 500 mg $FeCl_3$ in a mixture of $H_2SO_4$ (20 mL) and glacial acetic acid (10 mL); diluted to 50 mL by water.

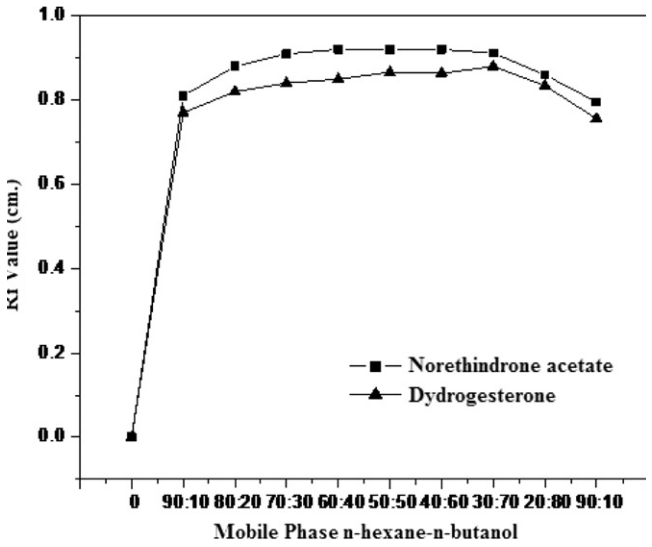


**Figure 2** The effect of mobile phase composition on $R_f$ values of norethindrone acetate and dydrogesterone on plain silica gel plates.

nated plates while it was yellow on plain plates. This behaviour is due to the complex formation of these drugs with copper metal ion.

### *3.2. Solid phase extraction (SPE)*

The recovered concentrations of norethindrone acetate and dydrogesterone were 0.079 and 0.073 mg from human plasma after 30 min. The percentage recoveries of these drugs were calculated and found to be 79% and 73%, respectively. These concentrations were calculated by applying the correction factors due to the blank experiments. The decrease in the concentrations of these drugs (21% of norethindrone acetate and 27% of dydrogesterone) may be due to their binding with plasma proteins. The optimization of SPE includes pH of phosphate buffer, flow rate of plasma sample and eluting solvents, and use of the different eluting solvents. SPE experiment was carried out with pH range of 1.0–8.0 and the maximum recoveries of these drugs were obtained at pH 2.0 for norethindrone acetate and at pH 3.0 for dydrogesterone. The different flow rates of plasma samples (0.10–0.50 mL/min.) were examined to optimize the SPE conditions and poor recoveries were obtained at high flow rate while it was maximum at low flow rate. The same situations were obtained with eluting solvents. The optimized flow rates for both plasma samples and eluting solvents were 0.10 mL/min. Attempts have been made to optimize SPE conditions by using different solvents, such as methanol, ethanol, ethyl acetate and dichloromethane. Besides, the optimization was also carried out by eluting the reported drugs with these solvents with and without different amounts of acetic acid or trifluoroacetic acid. As a result of an extensive experimentation the best eluting solvent was pure methanol for these drugs. It is due to the fact that the polarity of methanol is quite enough to elute these drugs from $C_{18}$ cartridge under the reported experimental conditions.

### *3.3. Interaction of drugs with plasma proteins*

The reported percentage recoveries clearly indicate that dydrogesterone binds more with proteins in comparison to

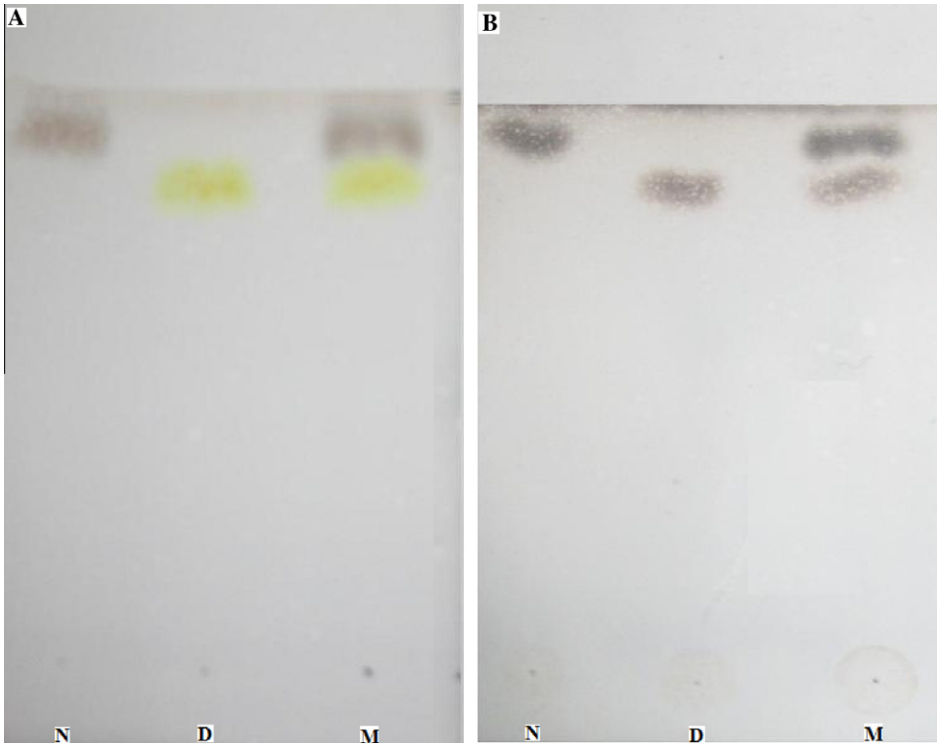


**Figure 3** Chromatograms of northindrone acetate and dydrogesterone on (A) plain silica gel layer and (B) Cu(II) impregnated silica gel layer. N: Norethindrone acetate, D: Dydrogesterone and M: Mixture of both drugs. Experimental conditions as given in Table 1.

norethindrone acetate. This may be due to the presence of more binding sites in former than in latter drug. Attempts have been made to verify and confirm the experimental binding results with the help of modelling by using PyMOL visualization tool and Autodoc4 softwares. Both drugs bind to the progesterone receptor forming the progesterone receptor binding complexes (Madauss et al., 2004). Norethindrone acetate and dydrogesterone are structurally similar except that norethindrone acetate and dydrogesterone have 22 and 23 non-hydrogen atoms (one extra $C_{19}$ methyl group and one extra double bond present at $C_6$–$C_7$ position of dydrogesterone; as shown in Figure 1). The carbonyl ketonic groups of both progestogens bind to the amino acid residues i.e. Gln725, Arg766 and Phe788 of progesterone receptor and water molecule (W) through hydrogen bonding and Van der Waals forces forming four bonds; as shown in Figure 4A and B. $C_{19}$ methyl group in dydrogesterone projects into a bubble like pocket in progesterone receptor providing Van der Waals forces while such a methyl group is not present in norethindrone acetate; lacking Van der Waals forces. Besides, $C_{20}$ methyl keto group of dydrogesterone also enhances Van der Waals interactions and hydrogen bonding with two nearest amino acid residues i.e. Asp 719 and Thr 894 of the progesterone receptor (Williams and Sigler, 1998). This situation causes more binding of dydrogesterone than norethindrone acetate (Madauss et al., 2004) to the protein and consequently the progesterone receptor. Besides, high affinity of dydrogesterone may be attributed to the presence of one extra double bond at $C_6$–$C_7$ position, which provides the rigid conformation to the molecule suitable for the interaction with the progesterone receptors (Colombo et al., 2006).

Norethindrone acetate has a larger group at $C_{17}$-α- (an ethynyl) and a smaller substituent at $C_{17}$-β- (a hydroxyl group). The $C_{17}$-β- group forms hydrogen bond with side chain of a residue of Asn719 (Madauss et al., 2004). Of course, a novel water mediated interaction between $C_{17}$-β-hydroxyl group of norethindrone acetate and Asn719 enhanced progesterone receptor binding affinity but our modelling suggests that norethindrone binds in the same orientation as dydrogesterone resulting into squeezing out of water molecule for optimal position and the hydrogen bond becomes weaker (Figure 4). In addition, dydrogesterone has one extra double bond,

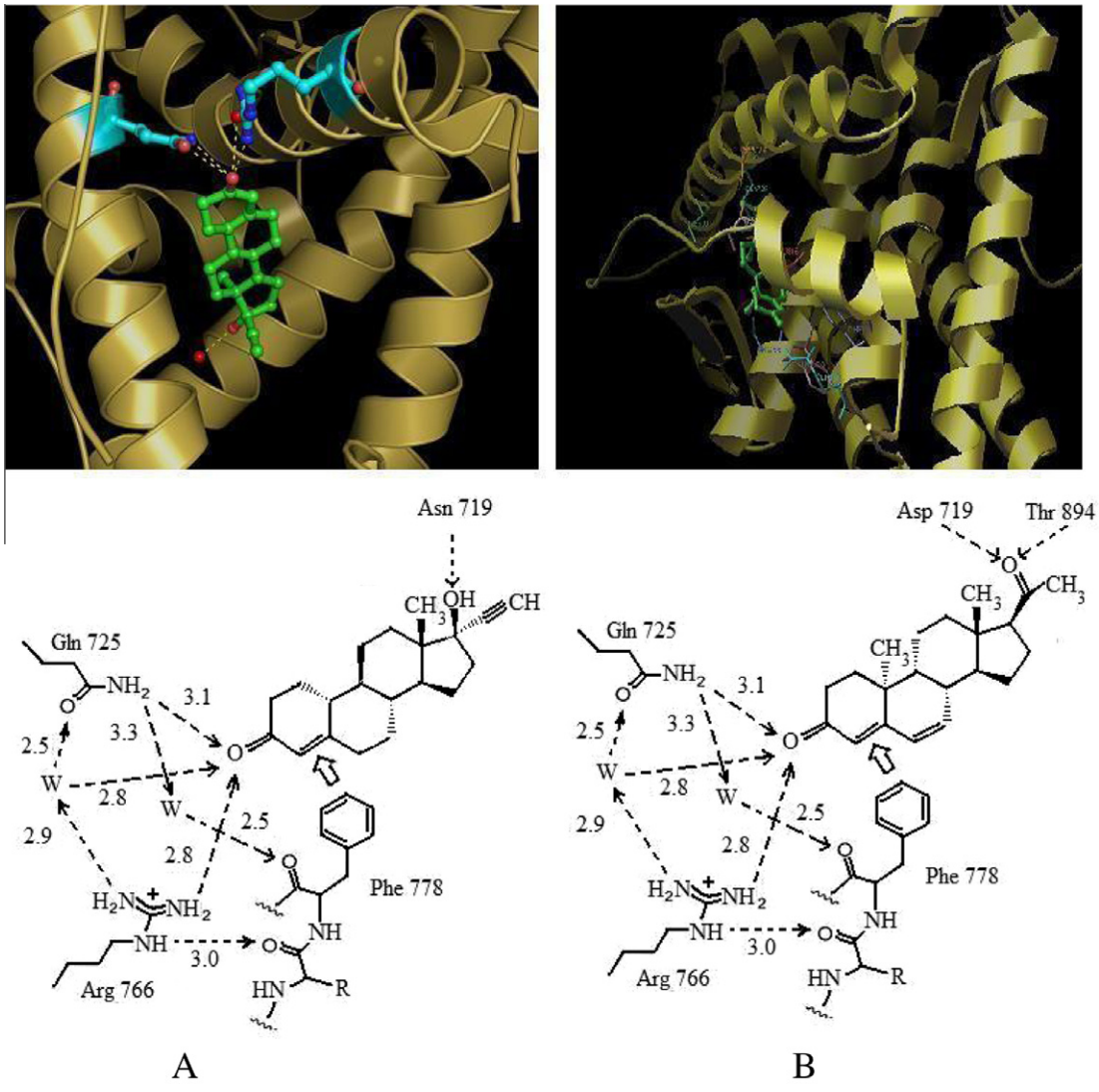


**Figure 4** Visualisation and chemical models of interactions of (A) norethindrone acetate and (B) dydrogesterone with progesterone receptor, respectively.

which provides interactions with phenyl ring of aromatic amino acids of progesterone receptor protein. The modelling visualization, analyses, comparison of all experimental results, all bindings and forces patterns indicate greater binding of dydrogesterone than norethindrone acetate.

## 4. Validation of the methods

The validation of the developed method was determined by carrying out five sets ($n = 5$) of the chromatographic and solid phase extraction procedures under the identical experimental conditions. The regression analysis was carried out using Microsoft Excel program. The standard deviation (SD) and correlation coefficients (R) for TLC were in the range of $\pm 0.040$–$0.041$ and 0.9997–0.9996, respectively, while the confidence levels varied from 99.4 to 99.5 for both contraceptives. Similarly, the values of standard deviation and correlation coefficients for SPE were from $\pm 0.070$ to $\pm 0.071$ and 0.9998 to 0.9996, respectively, for both drugs while the values of confidence level were 99.4 and 99.5 for norethindrone acetate and dydrogesterone, respectively. The correlation coefficients for calibration curves were higher than 0.997 as determined by least square analysis. The detection limits for norethindrone acetate and dydrogesterone were 0.40 and 0.10 ng, respectively.

## 5. Conclusion

The present article describes the determination of dydrogesterone and norethindrone acetate in plasma by using TLC–SPE methodologies. The results indicate that the developed method is inexpensive, reproducible, selective and efficient, which can be used to analyse these contraceptives in any blood samples. The detection limits are quite low, which is the requirement of today. The bonding patterns and mechanisms of these drugs have been developed and they clearly indicate higher binding of dydrogesterone than norethindrone acetate. This statement is being supportive by the fact that dydrogesterone is a more effective contraceptive than norethindrone acetate. The reported results are useful for further development of more effective oral contraceptives.

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