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Study of Clomiphene metabolism by LC/MS/MS

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Introduction

Clomiphene is an estrogen receptor modulator (SERM) sold as a mixture of stereisomers, enclomiphene and zuclomiphene [1]. Depending on the brand zuclomiphene percentage varies 30-50% [2].

These SERMs have mixed agonist and antagonist estrogenic activity, being zuclomiphene an estrogenic agonist and enclomiphene an estrogenic antagonist. The stereosisomers have different pharmacologic properties: enclomiphene is absorbed and eliminated faster then its stereoisomer [1-2].

In the last 30 years clomiphene was the most used drug in the female infertility treatment. This compound prevents the secondary effects of anabolic androgenic steroids abuse.

Several metabolites were detected by Ruenitz after i*n vivo* and *in vitro* clomiphene studies [3-6]. Two *m*-methoxy-*p*-hydroxy metabolites and unchanged clomiphene were detected in the feces after i.p. administration on immature female rats. Incubation of clomiphene in rat liver microsomal homogenate generates 4-hydroxy-clomiphene, 4´-hydroxy-clomiphene, 4´-hydroxy-3´-methoxy-clomiphene [3] and metabolites from N-oxidation and N-deethylation [4-5]. Incubation of enclomiphene with rabbit microssomes generated N-deethylenclomiphene and 4-hydroxy-clomiphene [6].

One hydroxylated metabolite had been reported in human urine after clomiphene administration.[7].

Reagents

17α-methyltestosterone and the enzyme sulfatase (Helix pomatia type H-2) were supplied by Sigma-Aldrich (St Louis USA). All chemicals (sodium acetate, acetic acid, terc-butylmethylether and methanol) were from Merck (Darmstadt, Germany), the enzymes β-Glucuronidase (E. coli) and β-Glucuronidase/Arysulfatase (Helix pomatia) were bought from Roche Diagnostics GmbH (Mannheim, Germany). All solvents for liquid chromatography (acetonitrile and formic acid) were from J.T.Baker (Deventer, Holland), water was from a MilliQ Gradient A10 system from Millipore (Molstheim Cédex , France).

Liquid Chromatography

An Alliance 2795 HPLC with quaternary pump and automatic injector (Waters, Millford, USA) was used to perform the experiences. Reversed phase chromatography was performed in a Xterra column (2,1x125 mm, 5μm) with a guard colunm Xterra (2,1x10 mm, 5μm). The mobile phase was acetonitrile/formic acid 0.1% (95:5, v/v) (A) and acetonitrile/formic acid 0.1% (5:95, v/v) (B) at a flow rate of 300μL. Gradient elution was as follows: the initial content was maintained at 25% of A for 1 min, increased to 40% in 9 min, to 80% in 5 min and maintained at this content 2 min, in 10 sec it went to the initial conditions of A and was maintained for 5 min.

Mass spectrometry

The liquid chromatograph was coupled to a triple quadrupole mass spectrometer (Quattro micro, Manchester, UK) with an ESI source.
Nebulisation and desolvation was made with nitrogen gas at the flow rates of 50 and 650 L/h respectively (nitrogen generator Maxigas N2MID600, Domnick Hunter, Dukesway, England) and argon as the collision gas. Source and desolvation temperatures were 100 and 400ºC. The analysis were performed in positive mode, with capillary at 3kV, the cone 60 V and the argon at 0,750 bar. Extracts were analysed in daughter scan and MRM modes in the following conditions:
1- Daugther scan of m/z 395.3 for N-dealkyl-hydroxy-clomiphene metabolites with cone at 60 V and collision energy 20 eV, for N-dealkyl-hydroxy-methoxy-clomiphene metabolites the

m/z 425.3 (cone 60 V, collision energy 30 eV), for hydroxy-clomiphene metabolites m/z 422.3 (cone 60 V, collision energy 20 eV), for hydroxy-methoxy-clomiphene metabolites m/z 452.3 (cone 60 V, collision energy 24 eV) and for methyltestosterone m/z 303 (cone 35 V, collision energy 22 eV).

2- The MRM conditions used to draw the elimination profile of the clomiphene metabolites are described in table 1.

Segments	Time (min.)	Transitions	Cone (V)	Collision energy (eV)	Dwell (s)
1	2-3.5	422.3> 80.1	60	22	20
		422.3> 95.1			
		422.3>404.3			
2	4.5-6.5	395.3>71.1	60	22	20
		425.3>71.1			
3	5.8-11	422.3>71.1	60	22	20
		422.3>85.1			
		422.3>99.1			
		452.3>71.1	60	20	20
		452.3>85.1			
		452.3>99.1			
4	12.4-14	303.3>97.1	35	22	20
		303.3>107.1			

Table 1. The MRM conditions

Metabolism study

An excretion study was performed after the administration of 50 mg of Dufine® to a 30 years old male that volunteered for the study. All urine samples were collected during the first day and spot urines were collected until day 4.

Sample preparation

Hydrolyse of the glucuronide and sulphate conjugates

To 3 mL urine 17α-methyltestosterone was added at a final concentration 100 ng/mL and the pH adjusted with 1 mL sodium acetate buffer 0.2 M pH 5.2 to carried out the hydrolysis with sulfatase (50μL) and β-glucuronidase/arysulfatase (50μL) or with 0,750 mL phosphate buffer 0.8 M pH 7 to hydrolise with β-glucuronidase (50μL). The sulphate conjugates were hydrolysed two hours at 55°C and the glucuronides one hour at the same temperature.

Isolation of the compounds of interest

The samples were centrifuged 10 min at 1680xg before being added to preconditioned (methanol and water) Oasis HLB (30 mg) cartridges. The column was rinsed with a mixture of 3 mL of aqueous solution of sodium hydroxy 0.02 M and methanol (6:4, v/v) and 3 mL of water.The columns were dried and then the compounds were eluted with 3 mL terc-butylmethyl ether and methanol (9:1, v/v). The eluates were evaporated under a stream of nitrogen and the residues reconstitute in 100 μL of the LC-eluent. Ten microliters of the samples were injected in the LC/MS/MS system.

Results and discussion

Screening of clomiphene

Metabolism of enclomiphene

For screening purpose the metabolites of enclomiphene should be monitored. The main metabolic reaction is oxidation in both phenyl rings, hydroxylation in *para* position and methoxylation in 3-*meta* position. The metabolites 4-hydroxy-enclomiphene and 3-methoxy-4-hydroxy-enclomiphene, produced in route 1 (figure 1) are eliminated as glucuronides and sulfate conjugates. The ratio is about 2:3 for the glucoconjugation. The main metabolite formed is the 4-hydroxy-enclomiphene and represents more then 50 % of the total metabolites produced in this excretion study. The metabolites 4´-hydroxy-enclomiphene and 3´-methoxy-4´-hydroxy-enclomiphene, produced via 2, are excreted mainly as glucuronides

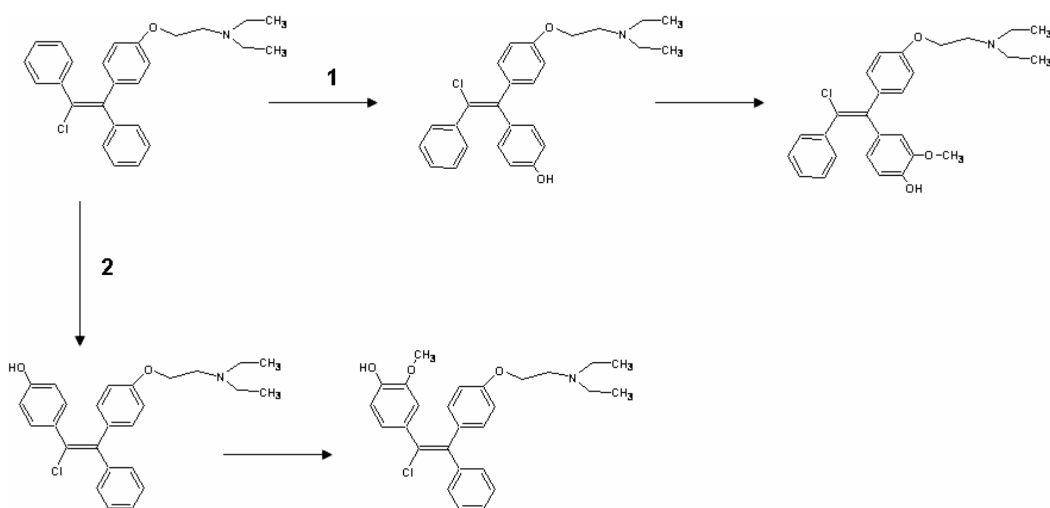

Figure 1. Metabolism of enclomiphene

The excretion profile of the enclomiphene metabolites (figure 2) shows that 4-hydroxy-enclomiphene is the major metabolite excreted during the first day. The 3-methoxy-4-hydroxy-enclomiphene is the main metabolite excreted in the following hours. The excretion profile as already described showed an entero-hepatic circulation of the metabolites. For screening purpose and with the aim to increase the time window detection both 4-hydroxy-enclomiphene and 3-methoxy-4-hydroxy-enclomiphene glucurono conjugated metabolites should be monitored.

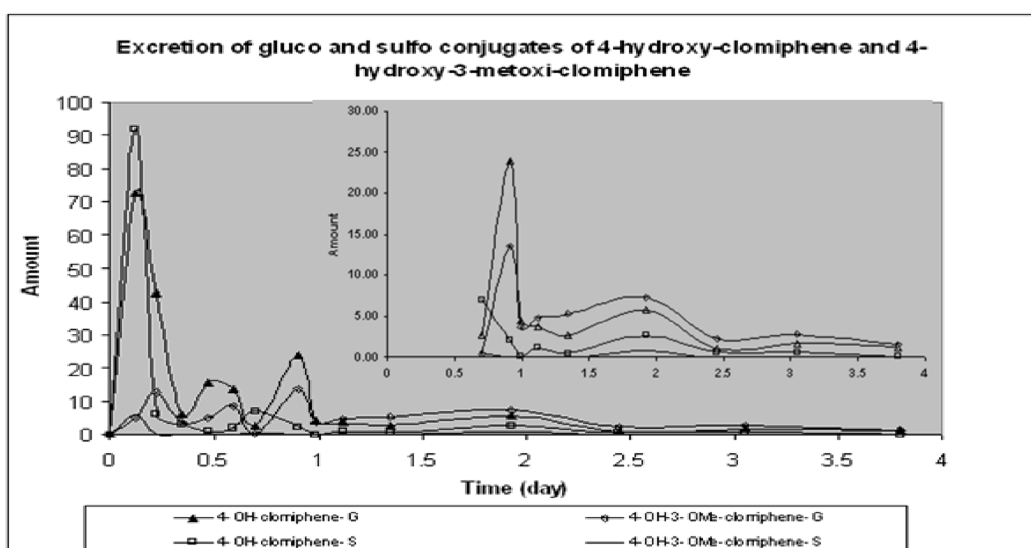

Figure 2. Excretion profile of enclomiphene metabolites gluco and sulfo conjugates

Mass spectra of clomiphene and metabolites

The enclomiphene biotransformation in the body was monitored by target fragments that indicates hydroxy and methoxy groups introduction in the parent structure. These target masses were chosen after clomiphene fragmentation pattern was identified.

Partial losses in the side chain of enclomiphene (figure 3) gave the product ions at m/z 100, 85, 72 and 57. The loss of the phenyl group followed by partials cleavages in the side chain gives the fragments at m/z 297, 281, 269 and 241. The loss 72 of mass gives the fragment at m/z 333.

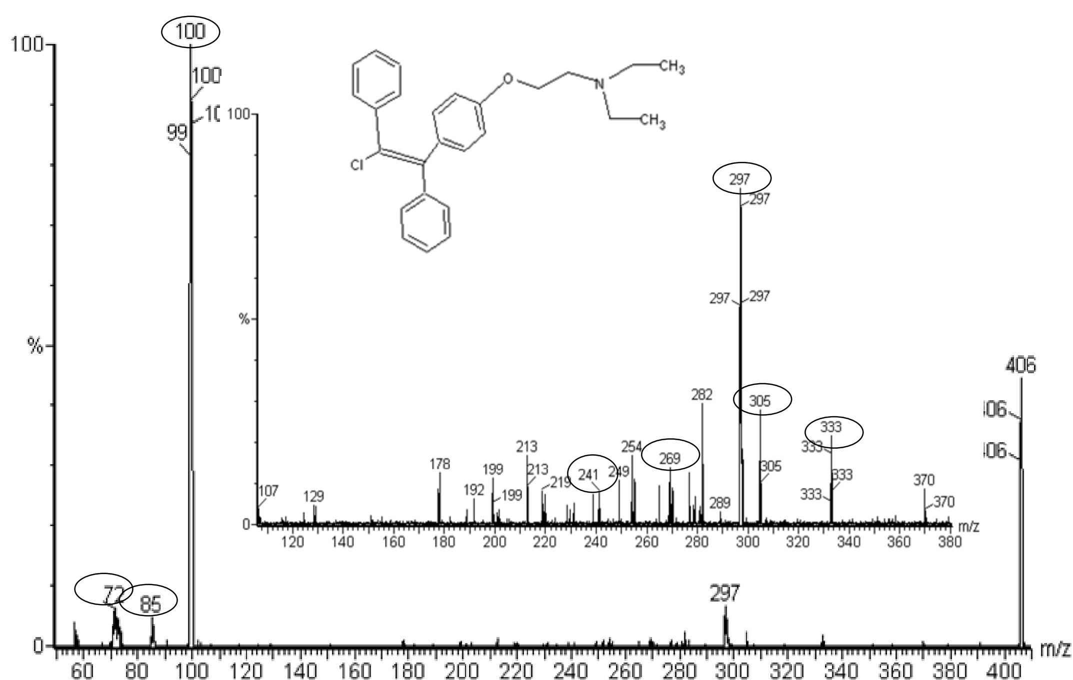

Figure 3. Mass spectrum of enclomiphene

The fragments of enclomiphene with the mass 297 and 269 were used as target fragments to monitor the introduction of one hydroxy group, masses 313 and 298, and the combine hydroxy and methoxy group represents 343 and 315 mass.

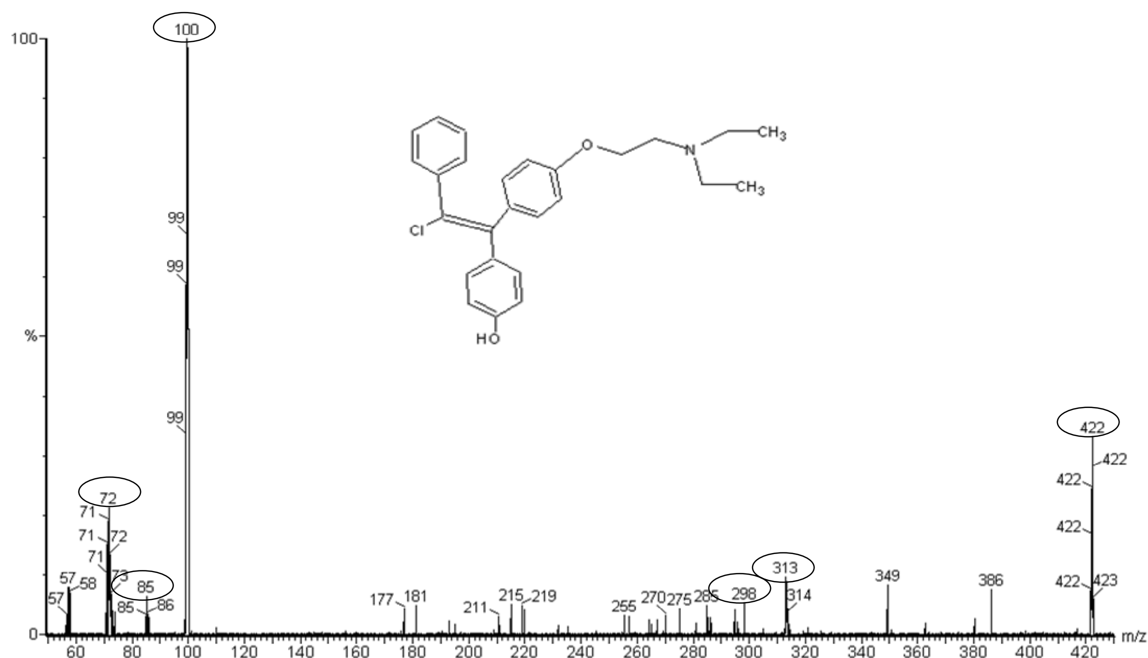

Figure 4. Mass spectrum of 4-hydroxy-enclomiphene

In the mass spectra of 4-hydroxy-enclomiphene (figure 4) and 3-methoxy-4-hydroxy-enclomiphene (figure 5) the target fragments are indicated. In the figure 6 two-proposed structures of the marked fragments are shown.

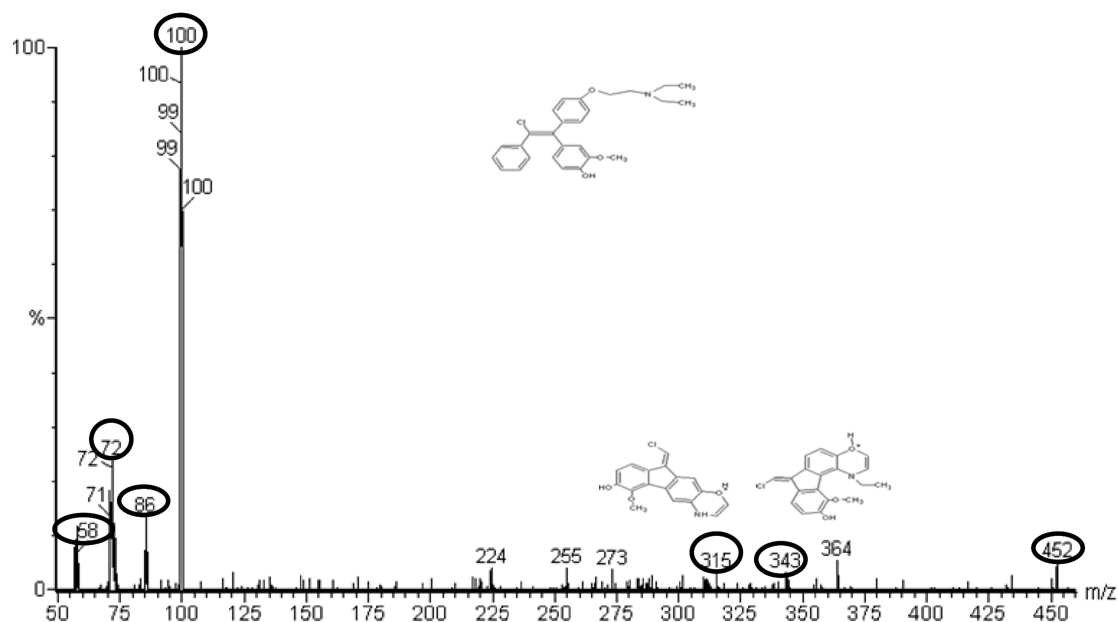

Figure 5. Mass spectrum of 3-methoxy-4-hydroxy-enclomiphene

A typical chromatogram of enclomiphene glucuronide metabolites is shown in figure 6.

The first metabolites to elute are the 4-hydroxy-enclomiphene and its methoxy metabolite and

then theirs regioisomers.

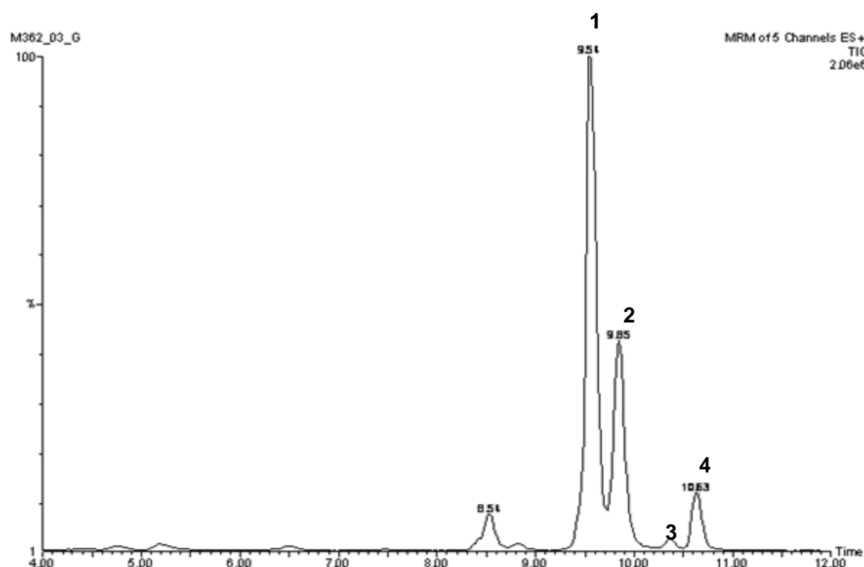

Figure 6. Chromatogram of enclomiphene metabolites gluco conjugated, (1)- 4-hydroxy-enclomiphene, (2)- 4-hydroxy-3-methoxy-enclomiphene, (3)- 4´-hydroxy-enclomiphene, (4)- 4´-hydroxy-3´-methoxy-enclomiphene

Confirmation of clomiphene

The identification of other stereisomer metabolites could be a useful tool for confimation purpose. Zuclomiphene undergo N-dealkylation and oxidation reactions (figure 7). Hydroxylation in the *para* position and methoxylation in the 3 and 5 *meta* position. All the metabolites are eliminated as sulfo-conjugates.

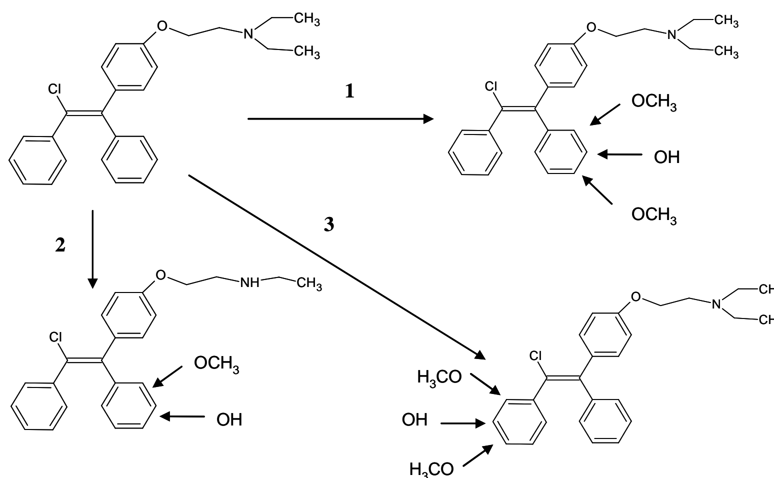

Figure 7. Metabolism of zuclomiphene

A typical chromatogram of zuclomiphene sulfate fraction is presented in the figure 8. Under the experimental conditions N-dealkylation metabolites elute first, followed by metabolites from the first route of metabolisation, (figure 7) and by metabolites from the third route.

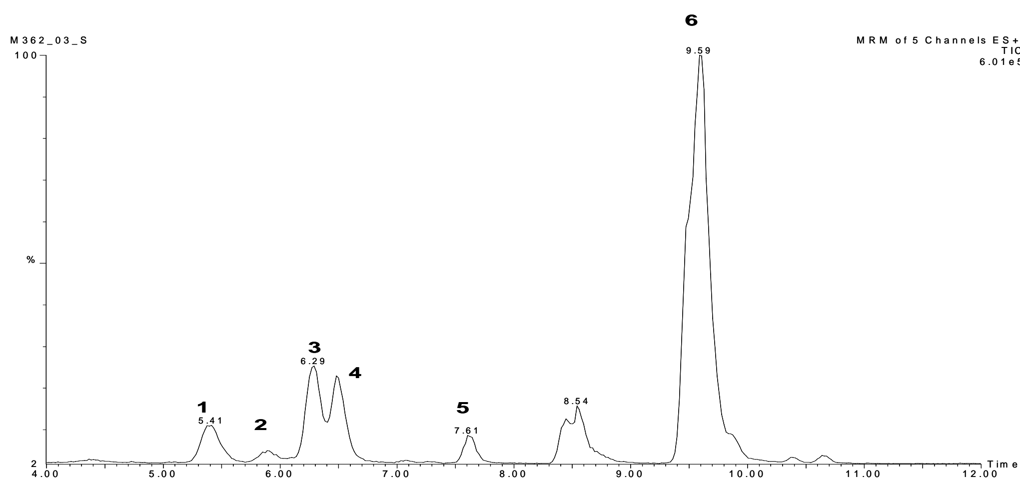

Figure 8. Chromatogram of zuclomiphene metabolites sulfoconjugate, (1)-N-deethyl-4-hydroxy-zuclomiphene, (2)- N-deethyl-4-hydroxy-3-methoxy-zuclomiphene, (3)- 4-hydroxy-zuclomiphene, (4 and 5)- methoxy-4-hydroxy-zuclomiphene, (6)- mixture of 4-hydroxy-enclomiphene and 4-hydroxy-3-methoxy-enclomiphene, 4´-hydroxy-zuclomiphene, and 4´-hydroxy-3´-methoxy-zuclomiphene

Conclusion

The 4-hydroxy-enclomiphene and 3-methoxy-4-hydroxy-enclomiphene, sulfo and gluco conjugates are the target compounds to be monitored in the screening. In this study 4-hydroxy-enclomiphene is the major metabolite formed.

Detection of sulfo conjugate metabolites of zuclomiphene could be useful for an unequivocal identification of clomiphene administration.

This stereoisomer undergoes several phase I reactions, oxidation on the phenyl rings and N-dealkylation. Phenyl oxidation gives hydroxylated and methoxylated compounds. N-deethyl-4-hydroxy-zuclomiphene and N-deethyl-4-hydroxy-3-methoxy-zuclomiphene are products of N-dealkylation.

References

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