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p-Toluenesulfonyl isocyanate as a novel derivatization reagent to enhance the electrospray ionization and its application in the determination of two stereo isomers of 3-hydroxyl-7-methyl-norethynodrel in plasma

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Abstract

In this paper, *p*-toluenesulfonyl isocyanate has been reported as a novel derivatization reagent with strong nuclephilic reactivity for the hydroxyl compounds. The derivatization for the two pharmacologically active 3-hydroxyl metabolites, 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel by *p*-toluenesulfonyl isocyanate can be accomplished in 2 min under room temperature. The offline derivatization procedure introduced an easily ionizable sulfonylcarbamic ester moiety to the metabolites. This greatly improved the analyte's sensitivity in negative electrospray ionization and enabled us to achieve the desired lower limit of quantitation at 100 pg/ml in plasma. Therefore, a sensitive high performance liquid chromatography-mass spectrometry (HPLC–MS) method for the analysis of the two stereo isomers was developed. The method had been validated to be accurate, precise, and sensitive, and can be used for the metabolism pharmacokinetic study of tibolone in human subjects.

Keywords: *p*-Toluenesulfonyl isocyanate; Derivatization

1. Introduction

Tibolone [7α, 17α-7-methyl-17-hydroxyl-19-norpregn-5(10)-en-20-yn-3-one], also called 7-methyl-norethynodrel or Org OD14, is a synthetic steroid used in the hormonal replacement therapy (HRT) for postmenopausal women since 1985 [1]. Clinical data indicate that tibolone produces the hormonal effects needed to treat climacteric symptoms and to prevent long-term effects of the menopause without stimulating breast and endometrial tissues [2,3]. Tibolone itself had weak binding affinities to the human estrogen, progesterone and androgen receptors, while its 3α-hydroxyl metabolite (3α-hydroxyl-7-methyl-norethynodrel, Org 4094) and 3β-hydroxyl metabolite (3β-hydroxyl-7-methyl-norethynodrel, Org 30126) binded solely to the estrogen receptor [4,5]. The structures of tibolone and its 3-hydroxyl metabolites have been shown in Fig. 1. The merits shown in its clinical profile have been thought of as the results of the tissue specific activity of tibolone and its main metabolites [6,7]. The typical oral dose contains 2.5 mg of tibolone per day, and the steady-state plasma concentrations of the two 3-hydroxyl metabolites were reported to be in the 0.5–15 ng/ml range [8]. Therefore, a sensitive bioanalytical method, capable of quantitating 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel down to the fmol/l range is needed to effectively evaluate the pharmacokinetic profiles of tibolone after oral administration.

3α-Hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel had been reported to be determined

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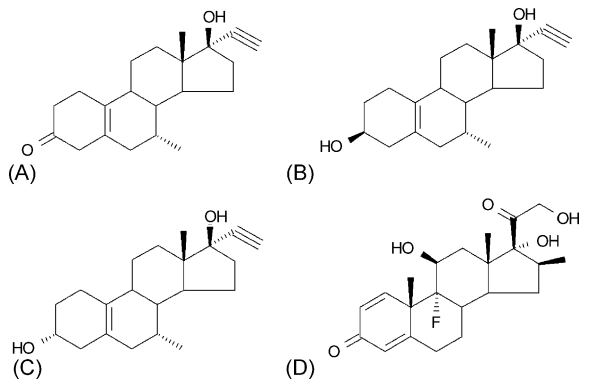

Fig. 1. Chemical structure of tibolone (A), 3α-hydroxyl-7-methyl-norethynodrel (B), 3β-hydroxyl-7-methyl-norethynodrel (C), and betamethasone (D).

by gas chromatography/mass spectrometry in the pharmacokinetic study of tibolone [8]. After solid phase extraction from plasma samples, the two metabolites were silanized and back-extracted by *n*-hexane. LOQ of the method was reported to be 0.1 ng/ml, while the sample pretreatment procedures, GC–MS conditions and method validation have not been reported yet.

Liquid chromatography coupled with atmospheric pressure ionization mass spectrometry (LC–API–MS) has become the method of choice for bioanalysis for steroids because of its high sensitivity and selectivity [9,10]. However, we found that 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel had rather low response using either atmospheric pressure chemical ionization or electrospray ionization due to their very non-polar nature. Chemical derivatization reagents, such as s-pentafluorophenyl tris(2,4,6-trimethoxyphenyl)phosphonium acetate bromide, mono(dimethylaminoethyl)succinyl imidazolide, 2-fluoro-1-methylpyridinium toluenesulfonate, ferrocenoyl azide, and 2-sulfobenzoic acid anhydride, have been reported in literature to improve the ESI-MS intensity of hydroxyl compounds [11–15]. These reagents can effectively improve the sensitivity of neutral analytes in ESI-MS by introduce either permanently charged moieties or easily protonated/deprotonated moieties to the analytes [16].

It is well known that oxygen atom possessed by hydroxyl has a weak nuclephilicity owing to its low electron density. Therefore, the derivatization procedures for hydroxyl compounds need either high temperature or long reaction period, which makes the derivatization not so convenient to be utilized [17,18]. Sulfonyl isocyanates are a series of compounds which have the general formula RSO_2NCO [18]. Owing to their strong nuclephilic reactivity, the reactions of sulfonyl isocyanates with hydroxyl compounds can be easily proceeded. To our knowledge, the application of sulfonyl isocyanates as derivatization reagents in HPLC or HPLC–MS analysis has not been reported.

In this paper, we reported *p*-toluenesulfonyl isocyanate as a novel derivatization reagent for hydroxyl compounds. Using this facile liquid phase derivatization procedure, an LC–ESI/MS bioanalytical method for the two 3-hydroxyl metabolites of tibolone was developed and validated to achieve an LOQ of 100 pg/ml in human plasma.

2. Experimental

2.1. Chemicals

The standards of 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel were both supplied by NV Organon (Oss, The Netherlands). The internal standard, betamethasone, was obtained from Shanghai Institute for Drug Control (Shanghai, China). *p*-Toluenesulfonyl isocyanate was purchased from Sigma–Aldrich (Dorset, UK). HPLC grade methanol and acetonitrile were obtained from Burdick & Jackson (Muskegon, MI, USA). Analytical grade ethyl acetate and ammonia acetate were purchased from SCR (Shanghai, China).

2.2. Extraction and derivatization

To 1 ml plasma placed in a 15 ml glass tube, 40 μl of betamethasone solution (100 ng/ml) was added. The sample was extracted with 6 ml ethyl actate for 2 min on a vertex mixer. After stationary and centrifuged for 5 min at 4500 × *g*, the organic layer was transferred into another clean tube and evaporated to dryness under a nitrogen stream with a water bath (45 °C). The residue was redissolved in 100 μl acetonitrile, and transferred to 1 ml glass vial. To each sample, 20 μl of *p*-toluenesulfonyl isocyanate solution (100 μl/ml in acetonitrile) was added followed by vertex-mixing for 2 min at room-temperature. Another 20 μl water was added to each sample to stop the derivatization. After vertex-mixing for 30 s, the sample was transferred to 100-μl conical vial for HPLC–ESI-MS analysis.

2.3. High performance liquid chromatography

The Agilent 1100 series HPLC sysem (Wilmington, USA) consisted of an on-line degasser, a binary pump, a column box and an autosampler. Seperation was achieved using a Diamonsil^{TM} C_{18} column (200 mm × 4.6 mm i.d., 5 μm) from DIKMA (Beijing, China) in combination with a guard column (Phenomix, USA), and the column temperature was set at 25 °C. The mobile phase consisted of two eluents, solvent A (0.2% amiamon actate in water, g/ml) and solvent B (methanol). The flow rate was 800 μl/min. A linear gradient elution was performed as follows: 0 min, 22% A, 78% B; 10 min, 100% B; 11–16 min, 22% A, 78% B. The volumn of injection onto the column was 80 μl, and the injector syringe was washed with methanol after each injection.

2.4. Mass spectrometry

The analytes were detected using an Agilent (Wilmington, USA) VL single quadrupole mass spectrometer equipped with an electrospray ionization source operated in the negative selected ion monitoring (SIM) mode. Operating conditions were optimized by flow injection analysis (FIA) and were determined as: drying gas (N_2), 13 l/min; dring gas temperature, 350 °C; nebulizer pressure, 40 psi; capillary voltage, 3000 V; fragmentator was set at 130 V. The specific mass range selected to monitor the derivatives of 3-hydroxyl metabolites and betamethasone were set at 510 (509.7–510.7) and 588 (587.7–588.7), respectively.

2.5. Method validation

Specificity of the method was evaluated by analyzing SIM chromatograms of drug-free plasma from six lots of different human plasma.

Calibration curves were prepared with drug-free plasma spiked with 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel both to cover the concentration range from 0.1 to 30.0 ng/ml and with the internal standard at the fixed concentration of 4 ng/ml. Calibration graphs were obtained by plotting drug concentrations against the peak–area ratio of metabolite versus betamethasone. The concentrations of the two metabolites in plasma samples were determined using the linear regression line with a weight of k/X^2 of the calibration standard.

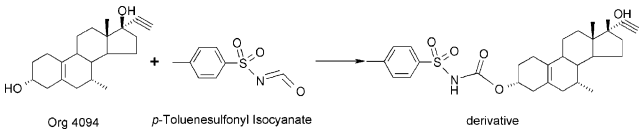

Fig. 2. Schematic representation for the chemical derivatization of 3α-hydroxyl-7-methyl-norethynodrel by *p*-toluenesulfonyl isocyanate.

The accuracy of the analytical method was determined by comparing the concentrations of the two 3-hydroxyl metabolites found from plasma through the calibration method to their spiked concentrations (0.25, 1.0 and 10.0 ng/ml, five replicates at every concentration).

Inter-day precision of the method was evaluated by analyzing drug-free plasma to which two metabolites had been added both at concentrations of 0.25, 1.0 and 10.0 ng/ml in five replicates. Intra-day precisions were evaluated over five different days at concentrations as mentioned above.

The extraction recoveries of the two metabolites in human plasma was determined by comparing the peak areas of extracted QC samples (at concentrations of 0.25, 1.0 and

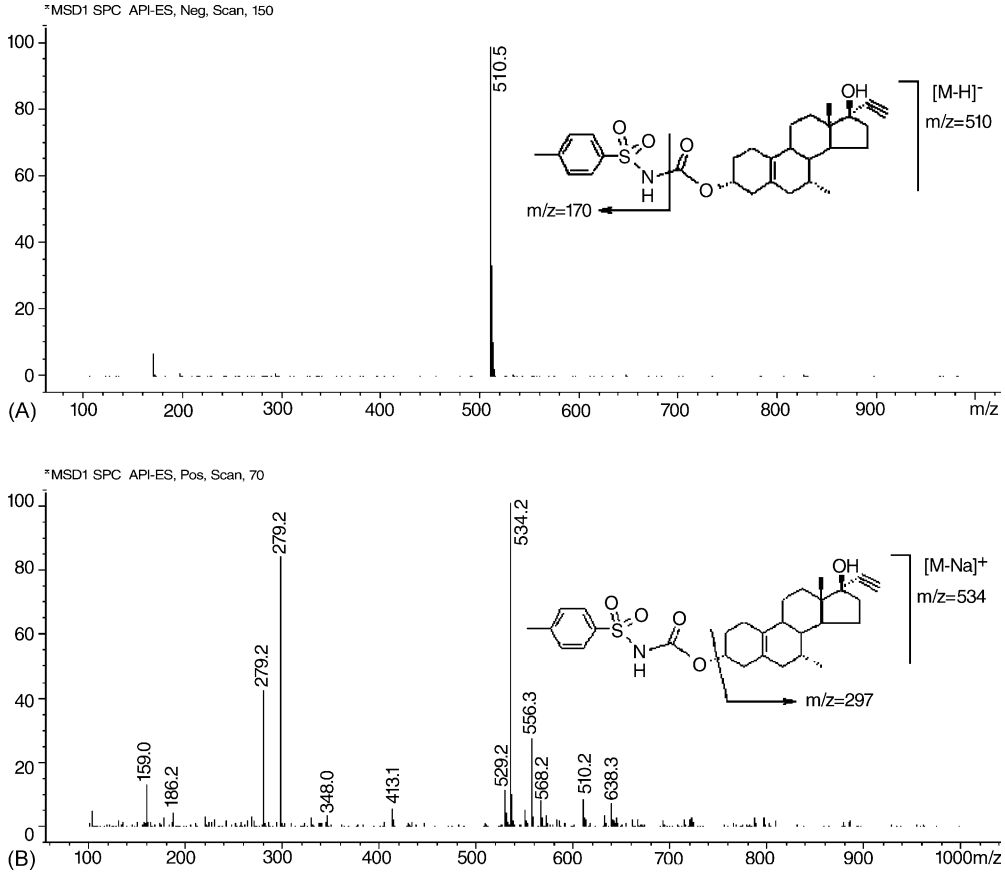

Fig. 3. Full scan mass spectrums of the derivative of 3α-hydroxyl-7-methyl-norethynodrel with *p*-teluenesulfonyl isocyanate by electrospray ionization in (A) negative mode and (B) positive mode.

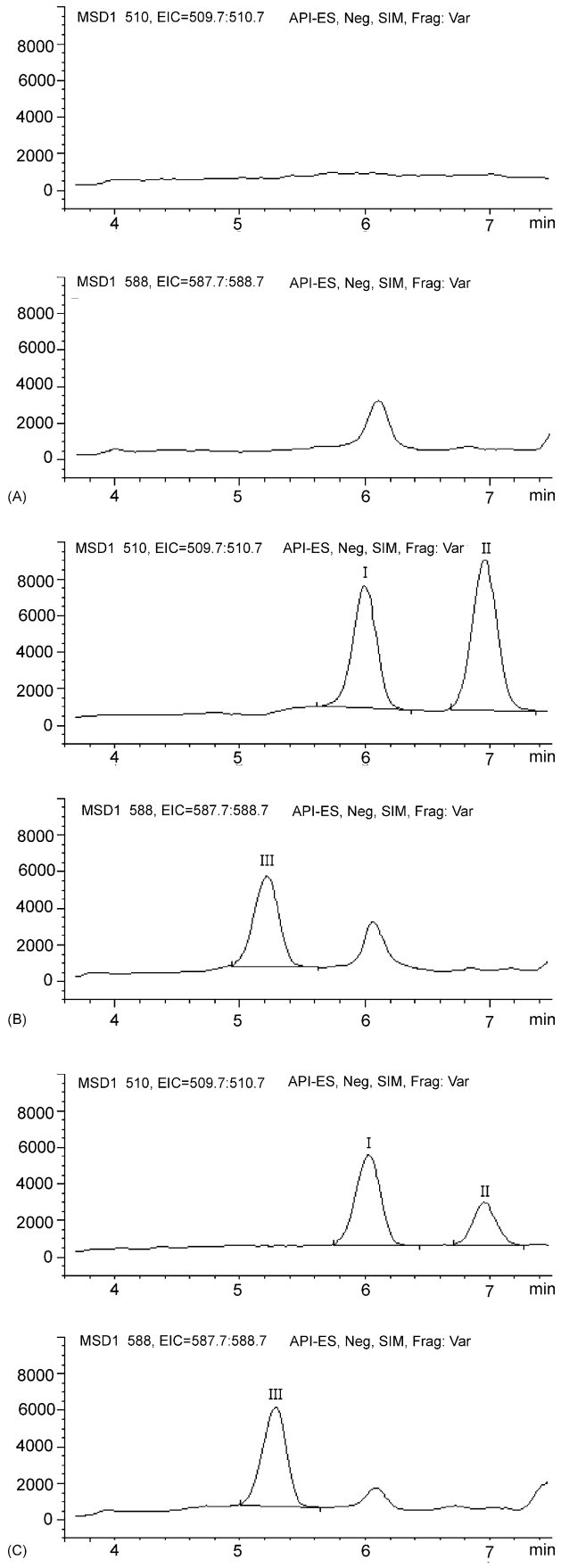

Fig. 4. LC/ESI-MS SIM chromatograms of (A) drug-free plasma sample, (B) drug-free plasma spiked with 2.5 ng/ml 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel, and (C) plasma sample from volunteer with a 3α-hydroxyl-7-methyl-norethynodrel concentration of 1.68 ng/ml and a 3β-hydroxyl-7-methyl-norethynodrel concentration of 0.69 ng/ml: peaks I–III refer to the derivatives of 3α-hydroxyl-7-methyl-norethynodrel, 3β-hydroxyl-7-methyl-norethynodrel and betamethasone (IS), respectively.

10.0 ng/ml) with the peak areas of recovery samples prepared by adding compound to post-extraction plasma blanks at the corresponding QC concentrations.

3. Results discussion

3.1. Derivatization

Our previous attempts to develop an LC–MS assay for 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel without derivatization failed. We found that the two metabolites had practically no response in negative ionization mode and low response in positive ionization mode using either atmospheric pressure chemical ionization or electrospray ionization. The lowest detection limit ($S/N > 3$) achieved for the two metabolites in working solution were both higher than 5 ng/ml by HPLC–ESI/MS. In sight of the weak ionization of the 19-norpregn core structure of the metabolites, we tried to introduce a highly ionizatable group into the molecules using a facile aqueous phase derivatization reaction.

It is well known that oxygen atom possessed by hydroxyl has a weak nuclephilicity owing to its low electron density. Therefore, the derivatization procedures for hydroxyl compounds need either high temperature or long reaction period, which makes the derivatization not so convenient to be utilized [17,18]. Sulfonyl isocyanates are a series of compounds with very strong nuclephilic activity. Owing to the polar sulfonyl group attached to the cumulative double bond system, the reactivity of the isocyanato group toward nucleophilic attack on the center carbon atom in sulfonyl isocyanate is vastly enhanced [19]. Therefore, the rate of reaction of sulfonyl isocyanates with alcohols is extremely fast [19,20]. In our study, we found that the derivatization reaction of *p*-toluenesulfonyl isocyanate with 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel can be accomplished in 2 min under room temperature, and generated *p*-toluenesulfonylcarbamic ester. The derivatization reaction scheme was shown in Fig. 2. Since sulfonyl isocyanates can react with water rapidly and quantitatively to generate sulfonamides [19], the excess derivative reagent were eliminate by hydrolysis.

The internal standard betamethasone has three hydroxyls in its C-11, C-17 and C-22 position, respectively. At first, we tried to use dexamethasone acetate as the internal stan-

dard, which has two hydroxyls in its C-11 and C-17 position. However, no chromatographic peak for the derivative of dexamethasone acetate with *p*-toluenesulfonyl isocyanate could be found in either DAD chromatogram or total ion current plot of ESI-MS. Further, we found that only one more peak existed in the chromatogram of derivatized betamethasone solution compared with blank derivatization solution. It may be related to the tension of the androstane ring that the C-11 and C-17 hydroxyl group in betamethasone and dexamethasone acetate could not be derivatized by *p*-toluenesulfonyl isocyanate easily.

3.2. Mass spectrometry analysis

Under the attractive effects of sulfonyl group and carbonyl group, hydrogen atom in imino group possessed by *p*-toluenesulfonylcarbamic ester can be easily dissociated. We discovered that the derivatives of the two metabolites had very high intensity both in positive and negative ionization mode. Mass spectrums of the derivatives of 3α-hydroxyl-7-methyl-norethynodrel with *p*-toluenesulfonyl isocyanate in both positive and negative ionization mode were shown in Fig. 3. Negative ionization mode has been chosen at last since there was lower chemical background in this mode.

3.3. Method validation

Under described chromatographic conditions, good resolution for the derivatives of the two stereo-isomers 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel was achieved. No endogenous substances interfered with the detection of the metabolites and internal standard in SIM–MS detection. Other xenobiotics possessed a hydroxyl, such as steroids, which may be coextracted and derivatized by *p*-toluenesulfonyl isocyanate, would not interfere with the detection of the two metabolites due to the high specificity of the gradient liquid chromatographic separation coupled with SIM–MS detection. Typical chromatograms of the drug-free plasma, metabolites standards and drug-free plasma spiked with standards after derivatization with *p*-toluenesulfonyl isocyanate have been shown in Fig. 4. No isomerization has been found during our study of the derivatization.

A linear calibration curve was constructed using the weighed (k/X^2) regression of the peak area ratio (3α-hydroxyl-7-methyl-norethynodrel/betamethasone) versus 3α-hydroxyl-7-methyl-norethynodrel concentration in plasma over the range of 0.1–30 ng/ml with a correlation efficient of 0.998 ($n = 9$). It confirmed the equation $y = 0.514C - 0.039$, where y refers to the area ratio of 3α-hydroxyl-7-methyl-norethynodrel to betamethsone. The calibration curve for 3β-hydroxyl-7-methyl-norethynodrel in plasma was also linear over the concentration range of 0.1–30 ng/ml with a weight of k/X^2, correlation efficient of 0.999 ($n = 9$). Its linear regression equation was $y = 0.613C - 0.063$, where y refers to the area ratio of 3β-hydroxyl-7-methyl-norethynodrel to betamethasone.

The lower limit of quantitation (LLQ) of the two 3-hydroxyl metabolites were both 100 pg/ml of plasma, with a signal-to-noise (S/N) ratio greater than 10. The mean accuracy for LLQ of 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel were 112 and 104%, as shown in Table 1. The inter- and intra-day precisions for LLQ of 3α-hydroxyl-7-methyl-norethynodrel were 10.7 and 13.8%, 9.6 and 11.3% for 3β-hydroxyl-7-methyl-norethynodrel. The overall precision for other QC samples ranged from 2.8 to 10.0% (Table 1). Fig. 5 showed the limit of detection (LOD) for the method of 20 pg/ml 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel in plasma at a signal-to-noise (S/N) ratio of 3.

The average extraction recovery of 3α-hydroxyl-7-methyl-norethynodrel in the three concentrations were between 70.5 and 79.9%, and the mean extraction recovery of 3β-hydroxyl-7-methyl-norethynodrel varied from 68.4 to 72.0%.

Table 1
The precision and accuracy for 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel determined by HPLC–ESI/MS

Nominal concentration (ng/ml)	Inter-day ($n = 5$)			Intra-day[a] ($n = 5$)		
	Measured concentration (ng/ml) (mean ± S.D.)	% RSD	% Accuracy	Measured concentration (ng/ml) (mean ± S.D.)	% RSD	% accuracy
3α-Hydroxyl-7-methyl-norethynodrel						
0.1	0.112 ± 0.012	10.7	112	0.116 ± 0.016	13.8	116
0.25	0.240 ± 0.015	6.3	96.0	0.281 ± 0.028	10.0	112
1.0	0.976 ± 0.040	4.1	97.6	0.916 ± 0.044	4.8	91.6
10.0	10.3 ± 0.3	2.9	103	9.42 ± 0.34	3.6	94.2
3β-Hydroxyl-7-methyl-norethynodrel						
0.1	0.104 ± 0.010	9.6	104	0.097 ± 0.011	11.3	97.0
0.25	0.240 ± 0.018	7.2	96.0	0.234 ± 0.023	9.8	93.6
1.0	0.988 ± 0.028	2.8	98.8	0.904 ± 0.080	8.8	90.4
10.0	10.3 ± 0.6	5.8	103	9.34 ± 0.57	6.1	93.4

[a] Over five different days.

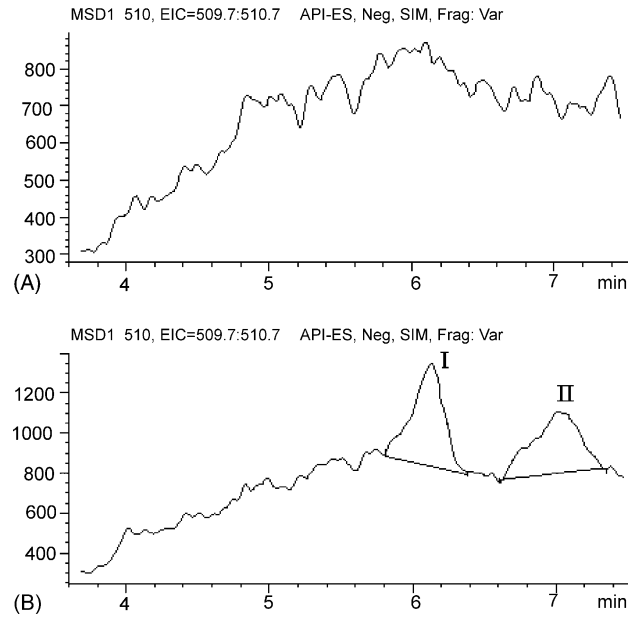

Fig. 5. LC/ESI-MS SIM chromatograms of (A) drug-free plasma sample and (B) LOD samples (20 pg/ml): peaks I and II refer to the derivatives of 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel, respectively.

4. Pharmacokinetic application

To evaluate the application of the proposed method in pharmacokinetic studies, a Chinese female human volunteer of 36 years old with a body weight of 56 kg was selected after giving written informed consent and having normal biochemical parameters. A Livial® tablet (containing tibolone 2.5 mg) was administered to the subject with 200 ml of warm water, and blood samples were obtained from an antecubetal vein prior to dosing and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, and 18.0 h after dosing were placed in heparinized tubes. The samples were immediately centrifuged at 3000 rpm for 15 min, and the plasmas were separated and frozen at −20 °C until analysis. The plasma concentration-time curves of the two 3-hydroxyl metabolites over 18 h were presented in Fig. 6. Following administration to the subject, the two metabolites both reach peak concentrations in blood at 1.5 h, while stereo-selectivity presented in peak concentrations and area under the curves (AUC_{0-T}) of 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel, 7.11 ng/ml versus 2.34 ng/ml of peak concentration and 7.09 ng h/ml versus 2.36 ng h/ml of AUC_{0-T}. A full metabolism pharmacokinetic study by this validated method of tibolone in female human volunteers is currently under way in order to evaluate the stereo-selectivity presented in the metabolism in vivo.

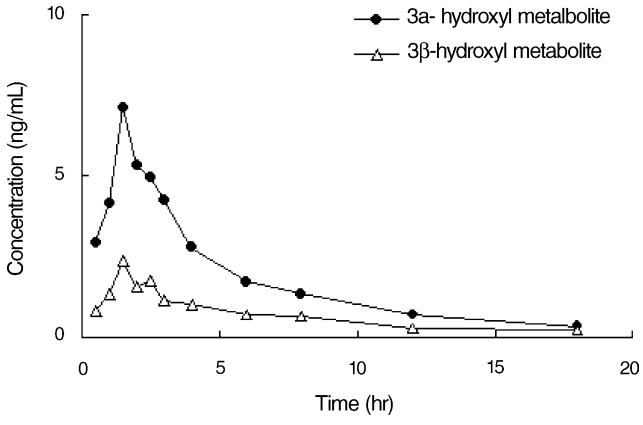

Fig. 6. Plasma concentration versus time profile of the 3α-hydroxyl-7-methyl-norethynodrel and 3β-hydroxyl-7-methyl-norethynodrel in a Chinese female volunteer dosed orally (0.25 mg) with tibolone.

5. Conclusions

p-Teluenesulfonyl isocyanate has been developed as a novel derivatization reagent for hydroxyl compounds in this study. The derivatization reaction can be accomplished in 2 min under room temperature without the existence of catalyse. A highly sensitive analytical method for the two pharmacologically active 3-hydroxyl metabolites of tibolone in human plasma was developed using LC–ESI/MS after derivatization by *p*-toluenesulfonyl isocyanate. The use of chemical derivatization greatly improved analyte's sensitivity in electrospray ionization, which in turn enabled us to achieve the desired lower limit of quantitation at 100 pg/ml. *p*-Toluenesulfonyl isocyanate has been demonstrated to be an attractive derivative reagent for hydroxyl compounds.

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