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Rapid communication

Determination of a new active steroid by high performance liquid chromatography with laser-induced fluorescence detection following the pre-column derivatization☆

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Abstract

A sensitive analytical method for the determination of a new active steroid, butane acid-(5-androsten-17-one-3β-ol)-diester (A1998), was developed by high performance liquid chromatography with laser-induced fluorescence detection following the pre-column derivatization with dansylhydrazine. The calibration curve for A1998 derivatization was found linear in the dynamic range from 0.025 to 5.0 μg/ml, with the precision less than 6% (CV) and the mean extraction efficiency greater than 92%. In 200 μl of plasma samples the limit of quantitation was as low as 0.025 μg/ml with a signal-to-noise ratio of 10. This assaying was further applied to the determination of the pharmacokinetic parameters of A1998 in rats with an intravenous injection of A1998. Values for clearance for elimination, volume of distribution at steady state and terminal half life in the above case were determined as 50.3 ± 1.1 ml/min kg, 1329.0 ± 111.0 ml/kg and 44.0 ± 2.7 min, respectively.

Keywords: Butane acid-(5-androsten-17-one-3β-ol)-diester; HPLC; Dansylhydrazine; Pharmacokinetic

1. Introduction

Butane acid-(5-androsten-17-one-3β-ol)-diester (A1998), as a derivative of dehydroxyepiandrosterone (DHEA), was synthesized by our group (Xu and Xu, 2003). Positive effects of A1998 were expected in extending both the action potential duration and effective refractory period because it can block the ultrarapid delayed rectifier K^+ current (I_{kur}) or transient outward K^+ current (I_{to}) (Xu and Xu, 2000). Previous studies suggested that A1998 has excellent antiarrhythmia activity (Zhu et al., 2003; Xu et al., 2004). In contrast to the most common anti-arrhythmia drug amiodarone, A1998 showed no mal-reactions including alveolar catarrh, pulmo-fibrosis, phosphatide depositing, thyrotumescence and drug depositing based upon long-termed toxic tests (Shukla et al., 1994). Therefore it is potential to develop A1998 as a novel class-III antiarrhythmial drug. For the purpose, we studied the pharmacokinetics and pharmacodynamics of A1998.

Experimentally the detection of A1998 was difficult. It is well known that mass spectrometry coupled with chromatographic separation is the most efficient detection technique for many steroids (Peng et al., 2002, 2006; Rauh et al., 2006; Yu et al., 2005; Seo et al., 2005; Impens et al., 2002). However, it is not suitable for A1998 since this species is hard to be aerosolized and ionized. High-performance liquid chromatography (HPLC) can provide alternative solutions for the determination of steroids in biological fluids (Główka et al., 2006; AbuRuz et al., 2003). But most of those reported methods involved complex solid-phase extraction procedures, requiring solid-phase extraction cartridges. In this paper, we explored the possibility of employing fluorescent derivatization prior to the HPLC separation. Compared with the traditional HPLC–UV methods, the selective fluoromophore labeling allows the detection of A1998 in a convenient but sensitive manner.

The fluorescent derivatization of A1998 is the key step and several factors including the fluorescent reagent, catalyst,

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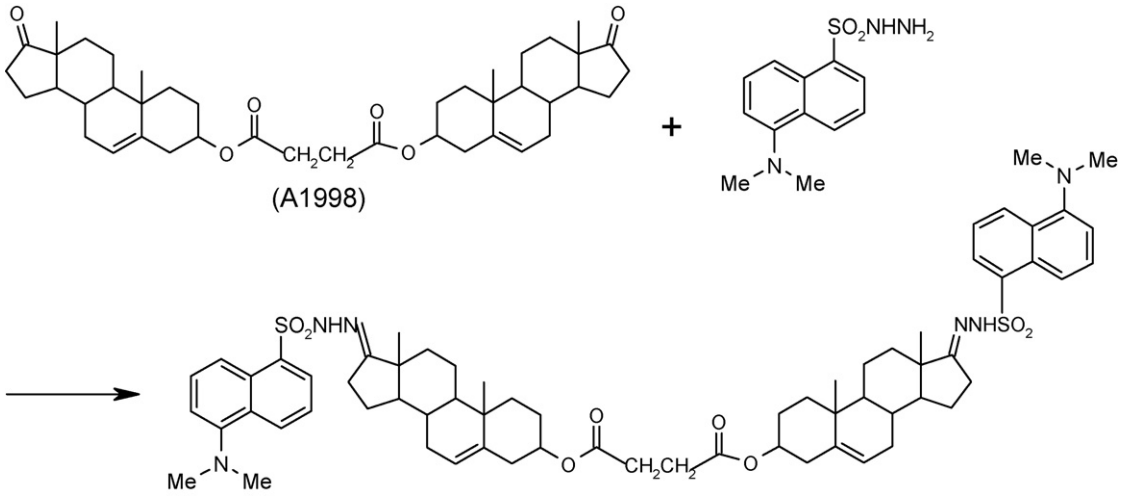

Fig. 1. The synthetic scheme of the A1998 derivative.

solvent, temperature and reaction time were taken into our consideration (Katayama et al., 1998; Hyytiaeinen et al., 1996; Appelblad et al., 2001; Saisho et al., 1998). In the present work, the optimal derivatization conditions were investigated. As expected, the bis-hydrazone derivatives significantly improved the detection limit of A1998 for the pharmacokinetic and bioavailability studies (Fig. 1).

2. Experimental

2.1. Chemicals and reagents

Dehydroepiandrosterone (DHEA), assay not less than 99%, was purchased from Sigma. Dansylhydrazine (DNSH, 5-dimethylaminonaphthalene-1-sulfohydrazide), trifluoroacetic acid (TFA) and trifluoromethanesulfonic acid (TFMSA) were obtained from Sigma. Butane diacid-(5-androsten-17-one-3-β-ol)-diester (A1998) and dehydroxyepiandrosterone acetate (DHEAAc) were prepared by the Lab of Traditional Chinese Medicine and Marine Drugs, School of Pharmaceutical Sciences, Sun Yat-Sen University. Tetrahydrofuran, methanol and acetonitrile were in HPLC grade and purchased from Fisher.

2.2. Animals

Female specific pathogen-free Sprague-Dawley were obtained from the Laboratory Animal Center of Guangdong Province, China, and fed in the Laboratory of Traditional Chinese Medicine and Marine Drugs, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangdong Province, China. Rats were kept in plastic cages in a room with a 12 h light/dark cycle at constant temperature (25 °C) and humidity (70%). Rats were allowed free access to tap water and pellets rodent diet. The rats for this study were handled in accordance with the Guidelines for Animal Experimentation of Sun Yat-Sen University.

2.3. Chromatographic system and conditions

The Agilent 1100 HPLC system was equipped with a G1311A QUATPUMP, a G1321A FLD, a 3395 integrator and a G1328A manual injector with a 20 μl loop (Agilent, USA). A Diamonsil C_{18} reverse-phase column (150 mm × 4.6 mm, 5 μm) from Dikma Technologies (Beijing, China) was used.

The mobile phase was consisted of a gradient mixture of acetonitrile and deionized water. The initial volume percentage of acetonitrile was increased to 98% from 75% after 12 min, and returned to 75% after an extra period of 8 min. The flow-rate was kept constant at 1 ml/min. The fluorescence was detected with the excitation and emission wavelengths of 350 and 520 nm, respectively.

2.4. Stock and working standard solutions

Dehydroxyepiandrosterone acetate (DHEAAc), an analog of A1998, can be well separated from A1998 by HPLC under the above chromatography conditions. DHEAAc was thus chosen as the internal standard (I.S.) in the assay of A1998. Stock solutions of A1998 and DHEAAc (3 mg/ml for each) were prepared in tetrahydrofuran. A series of A1998 solutions with the concentration range from 0.03 to 30 μg/ml were prepared from the stock solution using methanol. The DHEAAc was diluted to 40 μg/ml with methanol. The prepared solutions were stable at 4 °C for at least 3 months. Standard DNSH solution (8%, w/v in methanol) was freshly prepared and stored in a glass vial at 4 °C, which was wrapped with aluminium foil to avoid any possible photoreaction. TFA and TFMSA solutions were freshly prepared with methanol.

2.5. Sample preparation

Two hundred microliters of rat plasma and 50 μl of I.S. were mixed by standing on an orbital shaker for 30 s. Then 40 μl of $ZnSO_4$ solution (10%) and 2 ml of acetonitrile were introduced

to precipitate out plasma proteins. The suspension was vortexed for 5 min and centrifuged at 3000 × *g* for another 10 min. About 2 ml of the supernatant was transferred to another centrifuge tube and was evaporated to dryness at 40 °C using a nitrogen flow. The dry residue was redissolved with 200 μl of methanol. Then 100 μl of DNSH (8%, w/v) and 100 μl of TFA (20%, v/v) were added in consequence and the mixture was vortexed. The reaction was carried out in dark at room tempearture. Then the reaction mixture was centrifuged at 3000 × *g* and 20 μl of the supernatant was injected into the HPLC apparatus.

2.6. Validation

2.6.1. Linearity

The approach was evaluated by a calibration curve in the concentration range of 0.025–5.0 μg/ml of A1998 ($n = 5$). The plasma solutions were spiked with A1998 standard solutions to achieve the concentrations of 0.025, 0.05, 0.25, 0.5, 2.5, 5.0 μg/ml, respectively. The concentration of I.S. was 2.5 μg/ml in all cases. A calibration curve was obtained by plotting the peak area ratios of A1998 to I.S. versus the A1998 concentrations with least-squares linear regression analyses (Visich and Byron, 1996; Bland et al., 1990).

2.6.2. Precision and accuracy

The precision and accuracy of the replicates ($n = 6$) were evaluated by using three sets of concentrations of A1998, denoted as 0.025, 0.25 and 2.5 μg/ml, respectively. The precision and accuracy of the three sets were estimated from the results of three replicates of QC samples within 1 week. In specific, the precision was evaluated by the relative standard deviation (R.S.D.) and the accuracy of the method was examined by comparing the concentrations of spiked samples with the theoretical concentrations (Visich and Byron, 1996; Bland et al., 1990).

2.6.3. Recovery

The peak area ratios of A1998 after and prior to the extraction were used to evaluate the recovery of A1998 (Główka et al., 2006).

2.7. Pharmacokinetic application

The female Sprague-Dawley weighing 250 ± 20 g ($n = 7$) were placed in metabolic cages and fasted for 12 h prior to dosing. During the experiment, the rats were intravenously injected with A1998 (4 mg/kg). Serial blood samples (0.50 ml each) were collected at the time of 0, 1, 5, 15, 30, 45, 60, 120 and 180 min (7 rats/time points), and centrifuged for 10 min at 3000 × *g*. Two hundred microliters of the plasma were transferred to a centrifuge tube and store at −70 °C.

2.8. Pharmacokinetic analysis

The concentrations of A1998 in unknown samples were extrapolated from the calibration curve obtained. The data were subjected to the pharmacodynamics and pharmacokinetics analysis with the 3P97 software (Chinese Pharmacological Society). The concentration–time relationship was plotted and related parameters were listed on the graph as well.

3. Results and discussion

3.1. Derivatization conditions

The critical factors influencing the derivatization include molar ratio of reactants, catalyst, temperature, time and solvent. The influence of the molar ratio of dansylhydrazine to steroids has been investigated by other researchers (Appelblad et al., 2001). In this research a molar ratio of 1000 was used. As previously reported, the reaction time and temperature vary depending on the steroids structure, the catalyst and the reaction phase (Hyytiaeinen et al., 1996; Visser et al., 2000; Tomsova, 1991; Iwata and Suga, 1989). Hereby the efficiencies of two acidic catalysts, TFA and TFMSA, were compared.

3.2. Catalyst

Lewis acids such as hydrochloric acid, acetic acid, trifluoroacetic acid (TFA) and trifluoromethanesulfonic acid (TFMSA) are widely used as catalyst for the reaction, although there was a few report about using alkali catalyst (Peng et al., 2006; Rauh et al., 2006; Villareal and Holloszy, 2004; Ceschel et al., 2002). In particular, TFA abd TFMSA are very efficient catalysts for the derivatization of dansylhydrazine. Usually TFMSA is preferable for the fluorescent derivatization of sterone with polycarbonyl structure, because appropriate proportion of TFMSA can restrict the reactive site in 3-carbonyl position rather than the 17- and 20-positions, and lead to specific 3-hydrazone product with a higher yield (Yu et al., 2005). According to our experimental results, the yields are very close for both catalysts while the catalyst concentration is less than 1% (v/v). But the assay by HPLC indicated that the reaction catalyzed by TFA is a little slower than that of TFMSA, which took 16 h for the former and 12 h for the latter.

The influence of the catalyst concentration was also investigated (Fig. 2). It was revealed that the yield was significantly enhanced while the concentration of TFA was increased to 5%. A slight drop of the yield was found for further addition of TFA catalyst. On the contrary, an obvious decrease in the yield was noticed while the concentration of TFMSA was above 1%, which can be interpreted by the degradation of A1998 under strong

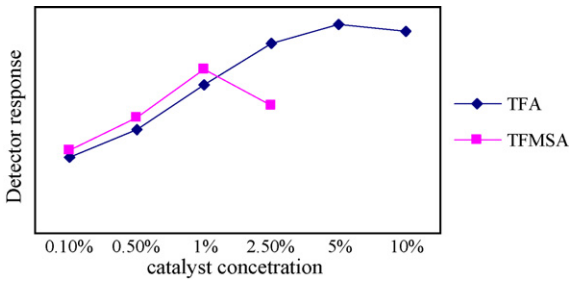

Fig. 2. Effect of catalyst concentration on the reaction yield: A1998 with a concentration of 2500 ng/ml was used at room temperature for 24 h.

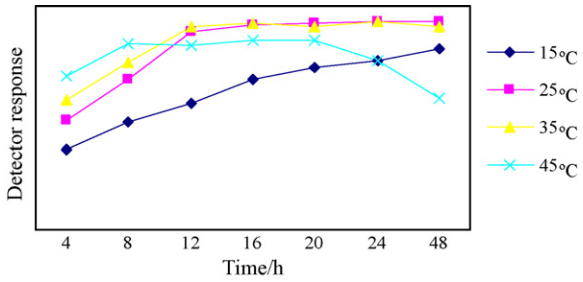

Fig. 3. Effect of the reaction time and temperature: A1998 with a concentration of 2500 ng/ml was employed.

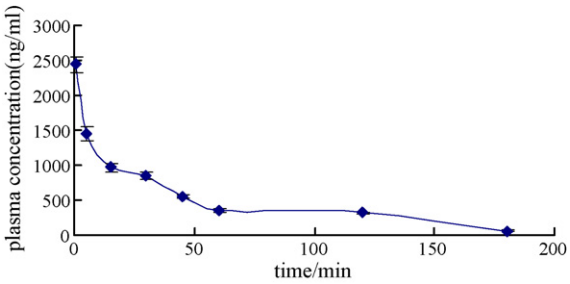

Fig. 5. Plasma concentration of A1998 4 mg/kg, following the intravenous injection to rats ($n = 7$, mean ± S.D.).

acidic conditions. Further experiments indicated that A1998 was complete degraded when the concentration of TFMSA exceeds 1%. Therefore, considering the fact of acidic degradation of A1998, 5% of TFA was adopted for the derivatization reaction.

3.3. Temperature and time

It was reported that the temperature and time of dansylhydrazations were of great differences by literatures. For instance, the reaction temperatures were from 20 to 60 °C and the reaction time from 0.5 to 20 h, respectively (Katayama et al., 1998; Appelblad et al., 2001; Visser et al., 2000; Al-Dirbashi et al., 2006; Du and Eddington1, 2002; Volpi, 2000; Claesona et al., 2001). These reaction conditions were usually relative to the different compounds, catalysts and reaction systems. So, the derivatization was performed at 15, 25, 35 and 45 °C, respectively, and the reaction time varied from 4 to 48 h. The results (Fig. 3) indicated that the reaction is slow at low temperatures such as 15 °C and the product is prone to degradation at relatively high temperatures such as 45 °C. Hence, the optimal conditions using 5% of TFA were 25 °C for 15–24 h.

3.4. Chromatography

According to the previous publications, the addition of buffer salt such as ammonium acetate into the mobile phase can improve the separation and peak symmetry (Katayama et al., 1998; Appelblad et al., 1997, 2001). However, A1998 derivatives usually have low polarity and needs a large proportion of organic solvent as mobile phase for HPLC separation. On the other hand, the buffer salt in the mobile phase will separate out and block the column while increasing the percentage of organic solvent due to their low solubility in most organic solvents. After examining several mixtures containing methanol (or acetonitrile) and water, a gradient mixture of acetonitrile and water was used as the mobile phase (Fig. 4). The peaks with the retention time of ca. 8.6 and 16.5 min can be safely assigned to the derivatives of A1998 and I.S., respectively.

3.5. Linearity

A linear dynamic range of the A1998 concentration was found from 0.025 to 5.0 μg/ml in rat plasma ($n = 5$). The mean regression-line was expressed as: A1998 (μg/ml) = $0.0020x + 0.0042$ ($r^2 = 0.9969$). Using 200 μl of plasma, the limit of quantitation for A1998 was determined as 25 ng/ml (signal-to-noise ratio = 10).

3.6. Precision, accuracy and recovery

Both the short-termed and long-termed precision and accuracy of measurements were summarized in Table 1. Both coefficients of variability were less than 6% in the dynamic range from 0.025 to 2.5 μg/ml. The mean recovery for A1998 in rat plasma was determined as 92.5 ± 5.7% at 0.05 μg/ml, 97.3 ± 7.0% at 0.5 μg/ml, and 99.4 ± 7.7% at 5 μg/ml ($n = 3$). The statistical results suggest that the proposed method is reproducible and reliable.

3.7. Pharmacokinetics in rat

The elaborated method was designed for pharmacokinetic studies on A1998 in vivo. Fig. 4C and D shows the representative chromatograms of rat plasma at 5 min and 120 min following an intravenous injection of A1998 at a level of 4 mg/kg. And Fig. 5 shows the curve of the mean plasma concentration versus time. Based on the time-dependence of plasma concentration, the pharmacokinetic parameters were calculated as shown in

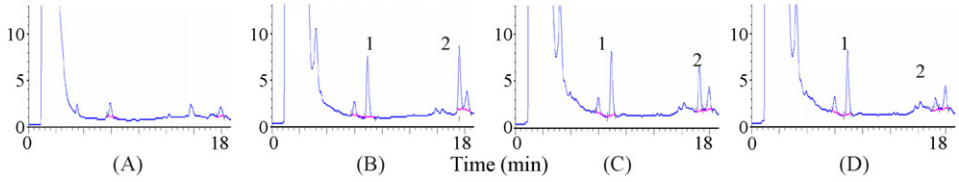

Fig. 4. Chromatograms obtained: (A) an extraction of blank plasma; (B) blank plasma spiked with A1998 (2500 ng/ml, peak no. 2) and internal standard DHEAAc (2500 ng/ml, peak no. 1); (C) rat plasma 5 min; (D) 120 min after receiving an infusion of A1998 with a dose of 4 mg/kg (A1998 concentrations were 1452 and 330 ng/ml, respectively).

Table 1
Intra-day and inter-day precision and accuracy of the assay for A1998 in rat plasma

Concentration (ng/ml)	Intra-assay			Inter-assay		
	Observed (ng/ml) (mean ± S.D., $n = 5$)	CV (%)	Accuracy (%)	Observed (ng/ml) (mean ± S.D., $n = 20$)	CV (%)	Accuracy (%)
25	24.2 ± 1.9	3.4	102.70	25.8 ± 1.3	4.9	96.78
250	256.6 ± 7.4	5.6	96.42	246.9 ± 14.2	5.8	101.22
2500	2437.7 ± 40.8	3.2	98.67	2567.6 ± 151.3	5.9	97.30

Table 2
Pharmacokinetic parameters of A1998 after intravenous injection of 4 mg/kg to seven rat

Dose (mg/kg)	4
n	7
Clearance (ml/min kg)	50.3 ± 1.1
Volume of distribution at steady state (ml/kg)	1329.0 ± 111.0
Elimination half life (min)	44.0 ± 2.7

Table 2. The mean values for clearance volume of distribution at steady state and elimination half-life were 50.3 ± 1.1 ml/min kg, 1329.0 ± 111.0 mg/kg and 44.0 ± 2.7 min, respectively.

4. Conclusion

In summary, we investigated the HPLC method for detecting a new anti-arrhythmia steroid-A1998 in rat plasma. The selective fluorophore labeling of A1998 greatly improved the sensitivity of detection. It was proven to be a convenient, accurate and reproducible method. And the promising prospect of applying this approach in the metabolism studies of A1998 in P450 and cells was suggested.

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