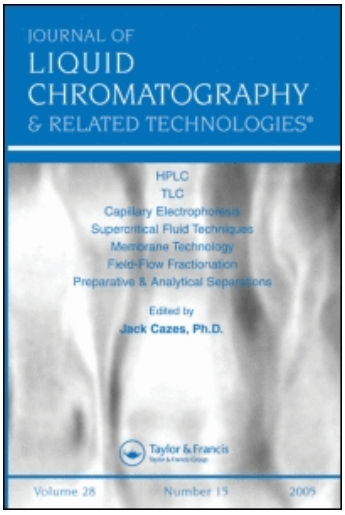

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Zou, Qiaogen , Huang, Biyun , He, Tingting , Wei, Ping , Zhang, Zunjian and Ouyang, Pingkai(2009) 'Identification, Isolation, and Characterization of Impurities in Sodium Tanshinone IIA Sulfonate', Journal of Liquid Chromatography & Related Technologies, 32: 16, 2346 — 2360
To link to this Article: DOI: 10.1080/10826070903187916
URL: http://dx.doi.org/10.1080/10826070903187916

Journal of Liquid Chromatography & Related Technologies®, 32: 2346–2360, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903187916

Identification, Isolation, and Characterization of Impurities in Sodium Tanshinone IIA Sulfonate

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Abstract: Six impurities in sodium tanshinone IIA sulfonate (STS) were observed by high performance liquid chromatography (HPLC) using DAD detector. One of the impurities, sodium tanshinone I sulfonate was reported in literature, other five impurities were unknown having not been reported previously. Initially, all the six impurities were identified by the liquid chromatography-mass spectrometry (LC-MS) data. After the recrystallization, enrichment and primary separation with silica column of the crude samples of STS, four imputies were isolated from the crude sample by preparative HPLC. Based on the spectral data (UV, MS, ^{1}H NMR and ^{13}C NMR), the structure of these impurities were characterised as, sodium methyl tanshinonate sulfonate (impurity I), sodium tanshinone I sulfonate (impuritiy II), sodium 1,2-dehydro tanshinone IIA sulfonate (impurity III) and sodium przewaquinone A sulfonate (impurity IV).

Keywords: Identification, Impurities, Isolation, LC-MS, Preparative HPLC, Sodium tanshinone IIA sulfonate

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INTRODUCTION

Sodium tanshinone IIA sulfonate (STS), chemically known as sodium (1,6,6-trimethyl-10,11-dioxo-7,8,9-trihydrophenanthro[1,2-b]furan)-yl-2-sulfonate, is a water-soluble derivative of tanshinone IIA, which is the main lipophilic component contained in *Salvia miltiorrhiza* known as 'Danshen' in traditional Chinese medicine. The clinical use of tanshinone IIA is limited by its poor water solubility, STS injection was thus developed. In China, STS injection have been used successfully for treatment of patients with coronary artery disease and angina pectoris for more than 30 years. STS has been shown to posses a wide spectrum of biological and pharmacological effects, including anti-platelet aggregation,[1,2] anti-oxidative[3] and anti-hypoxia activities,[4] acts against adriamycin-induced lipid peroxidation, attenuates hypertrophy induced by angiotensin II in cultured neonatal rat cardiac cells, protects ischemia-reperfusion injury through an electron transfer reaction in rat heart mitochondria against forming reactive oxygen radicals and blocks calcium channel.[5–8]

Literature available regards the determination of STS in biological samples by ion-pair reversed-phase HPLC method,[9,10] and liquid chromatography-electrospray ionisation-tandem mass spectrometry.[11] No literature is available regarding the analysis of STS and its impurities using chromatographic and spectroscopic methods till date to the best of our knowledge. As per International Conference on Harmonisation (ICH) guidelines for impurities in new drug substances, identification threshold is 0.1% and qualification threshold is 0.15% for maximum daily dose $\leq$2 g/day.[12] Hence, in order to meet the stringent regulatory requirements, it is important to identify and characterize the impurities in STS. This paper described the identification of impurities in STS, isolation by preparative HPLC and characterization of impurities using spectral data.

EXPERIMENTAL

Samples and Chemicals

Tanshinone IIA and tanshinone I was isolated from the root of *S. miltiorrhiza*, STS and impuritiy II was prepared from tanshinone IIA and tanshinone I, according to the method of Chien et al.[13] Their structure was identified by comparing the spectral findings with observations reported previously.[14] Impuritiy I, impuritiy III and impuritiy IV were isolated through preparative HPLC. HPLC-grade methanol was purchased from Tedia Inc. (Fairfield, USA). Water used was double distilled. Ammonium acetate and ethyl acetate and other chemicals and solvents used were all of analytical grade.

High Performance Liquid Chromatography

For identification of impurities, a Shimadzu LC-10Avp pump equipped with a Shimadzu SPD-10Avp detector (Kyoto, Japan) was used. The analysis was carried out on Symmetry C18, 150 mm long, 4.6 mm i.d., 5 μm particle diameter column (Waters, USA). A mixture of 0.05 M ammonium acetate (A) and methanol (B) in the ratio of 60:40 (v/v), at a flow rate of 1 mL/min was used as a mobile phase. The detection was carried out at 271 nm. The injection volume was 20 μL.

LC–MS Conditions

The electrospray ionization (ESI) and MS studies were performed using an Agilent 1100 Series ion trap spectrometer (Agilent Corp., USA) in negative ionization mode with the conditions as follows: the drying gas flow, 10 L/min; the drying gas temperature, 350°C; the nebulizer pressure, 40 pa; the fragmentor voltage, 70 V; Mass range was kept at m/z 100–800.

The chromatographic separation was carried out on an Agilent 1100 series quaternary gradient pump equipped with diode-array detector (DAD) and a degasser and an autosampler (Agilent Corp., USA). A Restek C18, 200 mm long, 4.6 mm i.d., 5 μm particle size column was used. The mobile phase consisted of 0.02 M ammonium acetate (A) and methanol (B), and an LC gradient method was developed for the separation of all possible related substances (impurities) of STS. Initial gradient starts with 50% of B and at 10 min it is 70%. The ratio being continued up to 15 min, at 30 min it is 95%, which is continued up to 45 min with a flow rate of 1.0 mL/min. Diluent was methanol. Six impurity peaks were detected in STS. The resolution mixture chromatogram, total ion current (TIC) plot and the mass spectrum is shown in Figs. 1–3, respectively. From mass values the structures given in Table 1 were suggested.

Preparative Liquid Chromatography Conditions

A AKTA explorer 100 system consisting of a P-900 pumping system and a UPC-900 detector (Amersham Pharamacia company, Sweden) was used. Hypersil 300A C18 (150 mm long, 20.0 mm i.d.) preparative column (Dalian Elite Analutical Instruments Co., Dalian, China) packed with 5 μm particle size was employed for isolation of impurities. The detection was carried out at 271 nm, 295 nm and 365 nm. Flow rate was 5 mL/min and the injection volum was 5 mL. The mobile phase consisted of 0.2 M

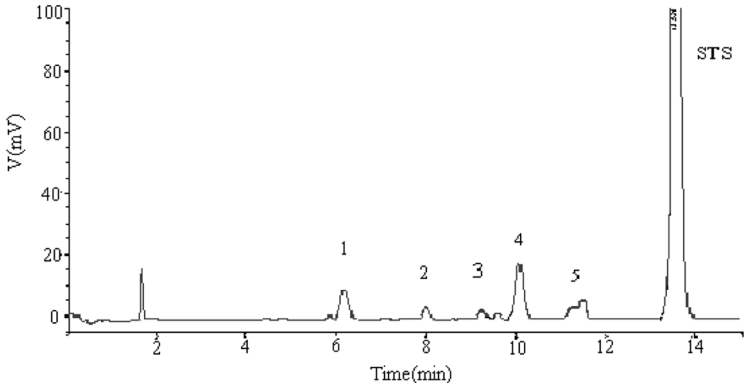


Figure 1. Typical LC-DAD chromatogram of STS sample and its impurities.

ammonium acetate (A) and methanol (B). Pump mode was gradient and was as follows.

For isolation of impurity I, time (min)/A (v/v): B (v/v); $T_{0.01}$/45:55, T_{10}/30:70, T_{15}/30:70, T_{30}/5:95, T_{45}/5:95.

For isolation of impurity II, time (min)/A (v/v): B (v/v); $T_{0.01}$/42:58, T_{10}/30:70, T_{15}/30:70, T_{30}/5:95, T_{45}/5:95.

For isolation of impurity III and impurity IV, time (min)/A (v/v): B (v/v); $T_{0.01}$/40:60, T_{10}/30:70, T_{15}/30:70, T_{30}/5:95, T_{45}/5:95.

UV

The UV spectra were recorded on Shimadzu UV-2401 PC double-beam spectrophotometer.

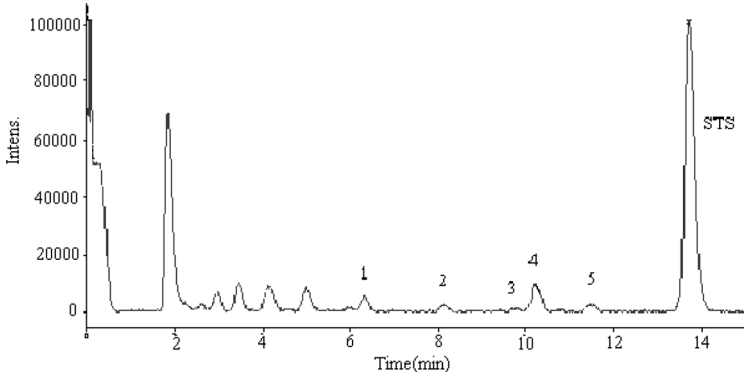


Figure 2. Typical TIC plot of TST sample spiked with impurities.

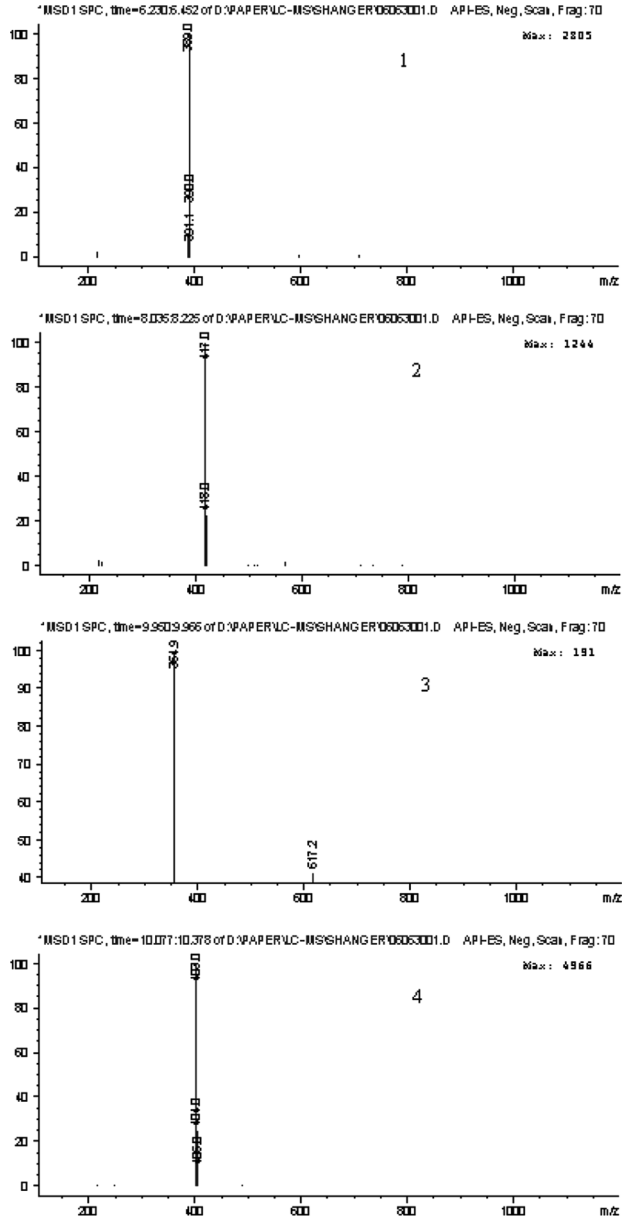


Figure 3. Mass spectrum of STS and its impurities. 1. Sodium tanshinone IIB sulfonate; 2. Sodium methyl tanshinonate sulfonate (Impurity I); 3. Sodium tanshinone I sulfonate (Impurity II); 4. Sodium salvianolic acid sulfonate; 5–1. Sodium 1,2-dehydro tanshinone IIA sulfonate (Impurity III); 5–2. Sodium przewaquinone A sulfonate (Impurity IV).

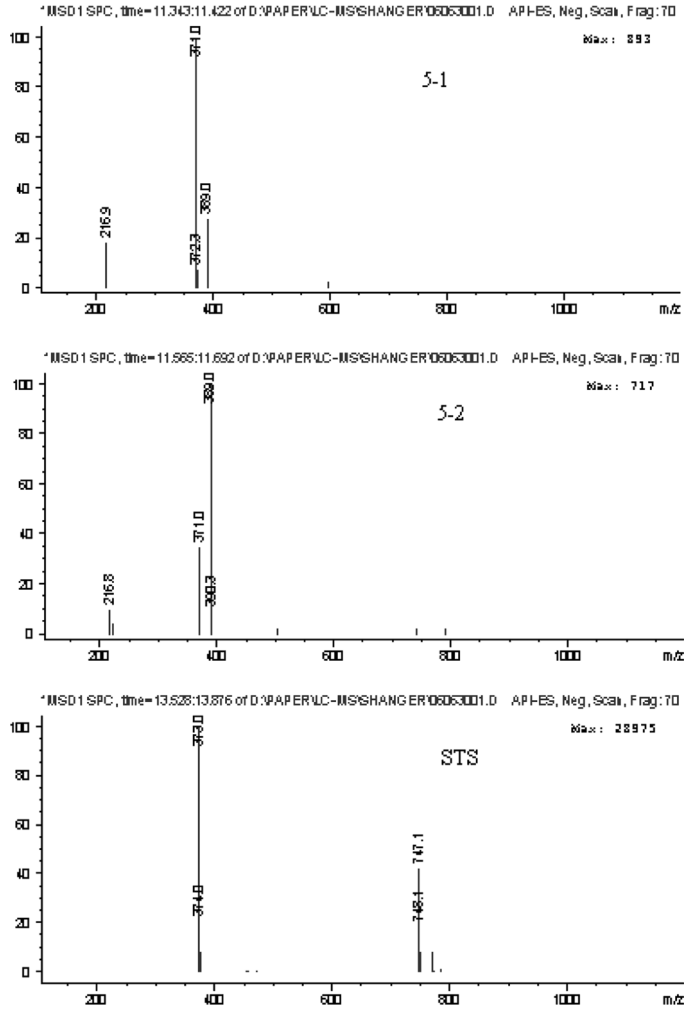
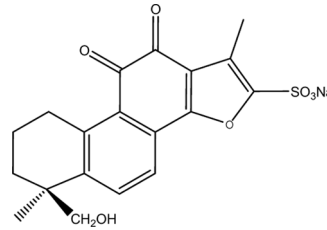
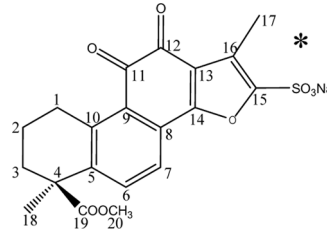
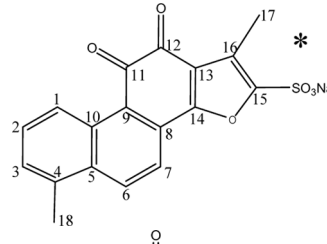
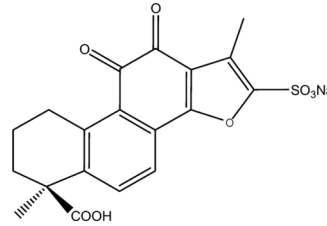
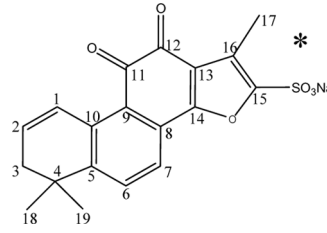


Figure 3. Continued.

NMR Spectroscopy

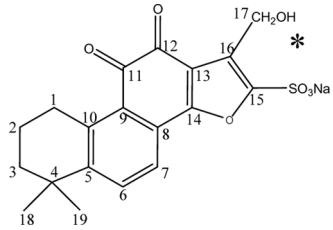
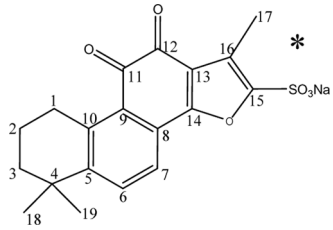
The ^{1}H NMR and ^{13}C NMR experiments were performed on a Bruker Avance DRX-500 MHz NMR spectrometer using deuterated dimethylsulfoxide (DMSO-d_6) as solvent and tetramethylsilane (TMS) as internal standard.

Table 1. LC Retention times, structures and molecular weights of the impurities and STS from LC-MS

S.no.	Retention time (min)	Compound	Molecular weight	Structure
1	6.23	Sodium tanshinone IIB sulfonate	412	
2	8.03	Sodium methyl tanshinonate sulfonate (Impurity I)	440	
3	9.96	Sodium tanshinone I sulfonate (Impurity II)	378	
4	10.07	Sodium salvianolic acid sulfonate	426	
5–1	11.34	Sodium 1,2-dehydro tanshinone IIA sulfonate (Impurity III)	394	

(*Continued*)

Table 1. Continued

S.no.	Retention time (min)	Compound	Molecular weight	Structure
5–2	11.56	Sodium przewaquinone A sulfonate (Impurity IV)	412	
STS	13.52	Sodium tanshinone IIA sulfonate	396	

*Structure numbering is for comparing ^{1}H NMR and ^{13}C NMR assignments, but not for chemical naming.

Isolation of Impurities by Preparative HPLC

After recrystallization with methanol repeatedly, the mother liquor of STS was concentrated and then loaded on a silica gel column using chloroform-ethyl acetate-methanol-formic Acid (4:4:1:0.01, v/v/v/v) as eluant. The separating flow chart was shown in Fig. 4. After primary separation, these impurities abtained were subjected for preparative isolation by using the conditions described in Section 2.4. Collected fractions were analyzed by analytical HPLC per the conditions mentioned in Section 2.2. And the chromatographic purities were 97.3% (impurity I), 95.8% (impurity II) and 96.0% (the mixtures of impurity III and impurity IV) by area normalization, respectively. Fractions of >90% chromatographic purity were pooled together, concentrated on rotavapour at 45°C to remove methanol. Concentrated fractions were passed through the preparative column by using water:methanol (50:50, v/v) as mobile phase to remove the buffer used for isolation. Again the eluate was concentrated using rotavapour to remove methanol. The aqueous solutions were lyophilized using freeze dryer (Virtis advantage 2XL). The impurity I, impurity II, mixture of impurity III and impurity IV, were obtained as nacarat, brown and dark red powders, respectively.

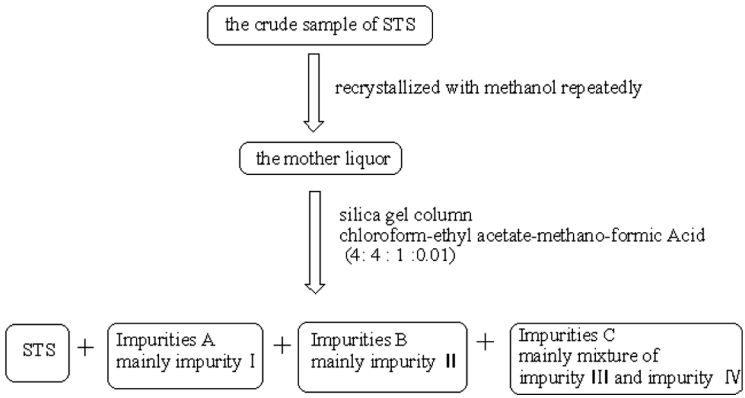


Figure 4. The separating flow chart of crude samples of STS.

RESULTS AND DISCUSSION

Detection of Impurity

The STS sample was dissolved using methanol as a solvent and then subjected for LC-MS analysis using the method described in Section 2.3. The resolution mixture chromatogram is shown in Fig. 1. Chemical structures of all impurities are shown in Table 1.

Structural Elucidation of Impurities

Impurity I (Sodium Methyl Tanshinonate Sulfonate)

The UV spectrum of impurity I was shown in Fig. 5, as is seen, the UV absorption maximum is 271 nm. ESI mass spectrum of sodium methyl tanshinonate sulfonate in negative ion mode showed a molecular ion peak at m/z 417 [(M-Na)$^-$] indicating the molecular weight of the compound as 440 which is 44 amu more than that of STS. Structurally there is a possibility of one more carbonyl group and oxygen than STS. To confirm this, ^{1}H and ^{13}C NMR spectra of isolated impurity were studied and compared to spectra of STS. In the ^{1}H NMR spectrum, it was observed that the methyl group, C(18)H3 had shifted from 1.29 ppm to 1.53 and an additional signal was observed at 3.61 ppm as singlet. In the ^{13}C NMR spectrum, additional signals were observed at 52.3 ppm and 176.3 ppm corresponding to –O–C20 and –C19=O, respectively. The HSQC spectrum displayed there is a methoxyl group in impurity I. Based on

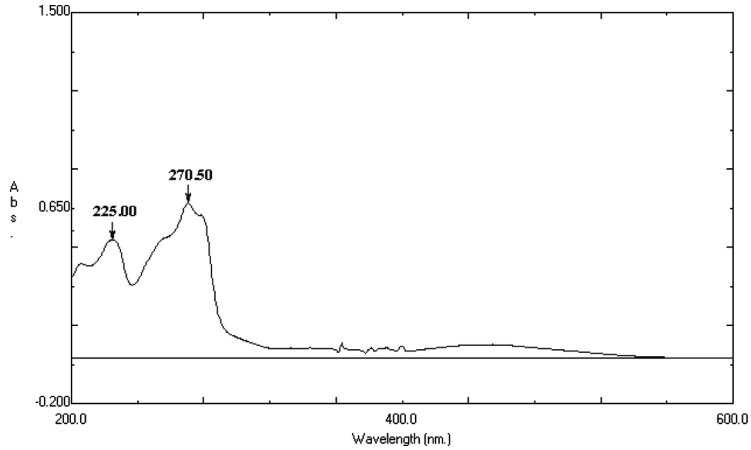


Figure 5. The UV spectrum of Impurity I.

the above spectral data the molecular formula of this impurity was confirmed as $C_{20}H_{17}O_8SNa$ and the corresponding structure was characterized as sodium (6 R-1,6-dimethyl-6-methoxycarbonyl-10,11-dioxo-7,8, 9-trihydrophenanthro[1,2-b]furan)-yl-2-sulfonate (sodium methyl tanshinonate sulfonate).

Impurity II (Sodium Tanshinone I Sulfonate)

For structural elucidation of impurity II, this impurity was prepared by the synthetic method. The UV spectrum (Fig. 6), retention time and m/z values of impurity II were correlated to the synthetically prepared material. ESI mass spectrum of sodium tanshinone I sulfonate in negative ion mode showed a molecular ion peak at m/z 355 [$(M\text{-}Na)^-$] indicating the molecular weight of the compound as 378 which is 18 amu less than that of STS. In the ^1H NMR spectrum, signals at 3.09 ppm, 1.73 ppm, 1.62 ppm and 1.29 ppm corresponding to C(1)H2, C(2)H2, C(3)H2, and C(19)H3 were disappeared and additional signals at 9.16 ppm, 7.60 ppm, 7.44 ppm and 2.69 ppm corresponding to C(1)H1, C(2)H1, C(3)H1, and C(18)H3 were observed. In the ^{13}C NMR spectrum, C19 at 31.4 ppm was absent and C1, C2, C3, and C18 had shifted from 29.4 ppm, 18.6 ppm, 37.3 ppm, and 31.4 ppm to 123.8 ppm, 130.0 ppm, 127.8 ppm and 19.2 ppm, respectively. Based on the above spectral data the molecular formula of this impurity was confirmed as $C_{18}H_{11}O_6SNa$ and the corresponding structure was characterized as

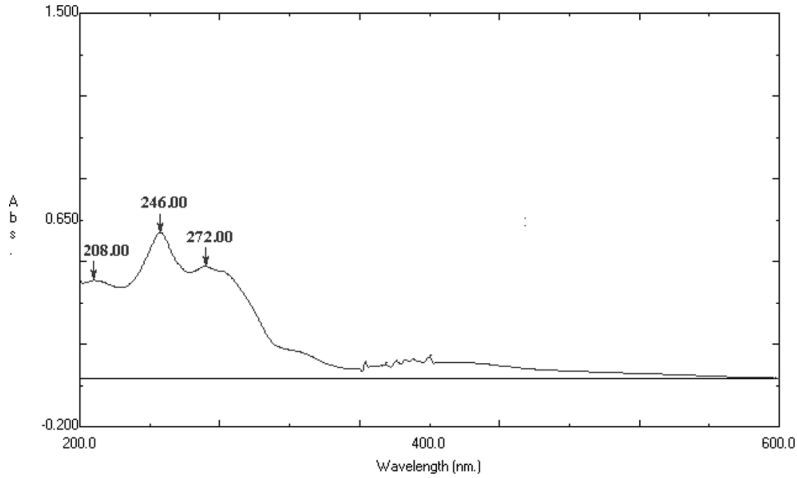


Figure 6. The UV spectrum of Impurity II.

sodium (1,6-dimethyl-10,11-dioxo-Phenanthro[1,2-b]furan)-yl-2-sulfonate (sodium tanshinone I sulfonate).

The Mixture of Impurity III and Impurity IV (Sodium 1,2-dehydro Tanshinone IIA Sulfonate and Sodium Przewaquinone A Sulfonate)

The UV spectrum of impurity III and impurity IV was shown in Fig. 7, as is seen, the UV absorption maximum is 253 nm. ESI mass spectrum of this sample in negative ion mode showed two molecular ion peaks at m/z 371 [(M-Na)$^-$] and m/z 389 [(M-Na)$^-$], respectively. It suggestes that the sample may be existed of two compounds, therein, the molecular weight of sodium 1,2-dehydro tanshinone IIA sulfonate as 394 which is 2 amu less than that of STS and the molecular weight of sodium przewaquinone A sulfonate as 412 which is 16 amu more than that of STS.

For structural elucidation of the sample (mixture of sodium 1,2-dehydro tanshinone IIA sulfonate and sodium przewaquinone A sulfonate), it was detected with ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, and HMBC spectra. The total number of protons observed in ^{1}H NMR spectrum were 46 protons except active hydrogen, moreover, there were a group of signals similar to STS and an additional singlet at 4.71 ppm. It is suggested that there is a compound similar to STS and a –OCH_2 group. The sample maybe consisted of impurity III and impurity IV (2:1, n/n), therein, the number of protons in impurity III and impurity IV is 15 and 16 except active hydrogen, respectively.

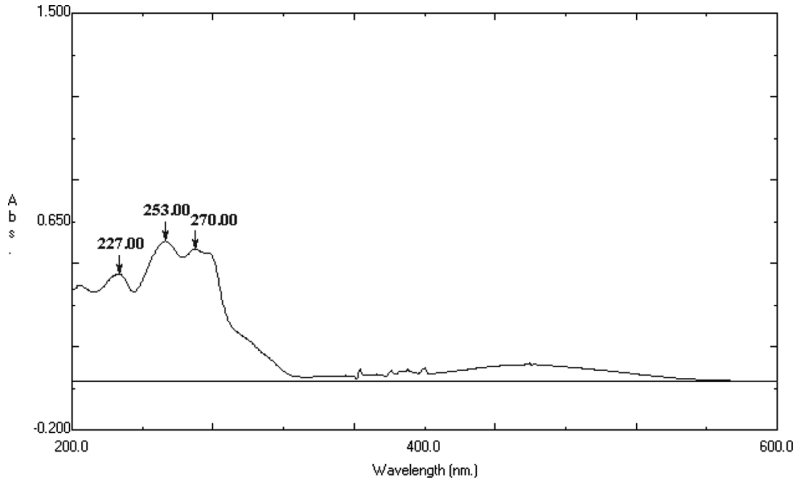


Figure 7. The UV spectrum of Impurity III and Impurity IV.

For impurity III: In the ^{1}H NMR spectrum, the signals at 1.73 ppm and 3.09 ppm corresponding to C(1)H2 and C(2)H2 were disappeared and additional signals at 6.35 ppm and 7.77 ppm corresponding to C(1)H1 and C(2)H1 were observed. In the ^{13}C NMR spectrum, C(1) and C(2) had shifted from 29.4 ppm and 18.6 ppm to 124.1 ppm and 133.5 ppm, respectively. From DEPT, COSY, HSQC, and HMBC it was confirmed as alkene CH_2 signal.

For impurity IV: In the ^{1}H NMR spectrum, the signal at 2.34 ppm corresponding to C(17)H3 was disappeared and an additional signal at 4.71 ppm corresponding to C(17)H2-O was observed. In the ^{13}C NMR spectrum, C17 at 9.2 ppm was absent and an additional signal at 53 ppm (O-C17) was observed. From DEPT, COSY, HSQC, and HMBC it was confirmed as alkene OCH_2 signal.

Based on the above spectral data the molecular formula of impurity III was confirmed as $C_{19}H_{15}O_6SNa$ and the corresponding structure was characterized as sodium (1,6,6-trimethyl-10,11-dioxo-7-hydrophenanthro[1,2-b]furan)-yl-2-sulfonate (sodium 1,2-dehydro tanshinone IIA sulfonate). The molecular formula of impurity IV was confirmed as $C_{19}H_{17}O_7SNa$ and the corresponding structure was characterized as sodium (1-hydroxymethyl-6,6-ditrimethyl-10,11-dioxo-7,8,9-trihydrophenanthro[1,2-b]furan)-yl-2-sulfonate (sodium przewaquinone A sulfonate).

The ^{1}H and ^{13}C NMR chemical shift values of STS and the impurities identified are given in Tables 2 and 3 respectively.

Table 2. Comparative ^{1}H NMR assignments for STS and the impurities identified

H[a]	STS ppm, multiplicity	Impurity I ppm, multiplicity	Impurity II ppm, multiplicity	Impurity III ppm, multiplicity	Impurity IV ppm, multiplicity
1-H	3.09 (t, 2H)	3.13 (m, 2H)	9.16 (d, 1H)	7.77 (d, 1H)	3.10 (t, 2H)
2-H	1.73 (m, 2H)	1.74 (m, 2H)	7.60 (t, 1H)	6.35 (m, 1H)	1.74 (m, 2H)
3-H	1.62 (t, 2H)	2.15 (m, 2H)	7.44 (d, 1H)	2.26 (dd, 2H)	1.63 (m, 2H)
6-H	7.84 (d, 1H)	7.61 (d, 1H)	8.49 (d, 1H)	7.72 (d, 1H)	7.85 (d, 1H)
7-H	7.56 (d, 1H)	7.57 (d, 1H)	7.84 (d, 1H)	7.57 (d, 1H)	7.58 (d, 1H)
17-H	2.34 (s, 3H)	2.32 (s, 3H)	2.36 (s, 3H)	2.32 (s, 3H)	4.71 (S, 2H)
18-H	1.29 (s, 3H)	1.53 (s, 3H)	2.69 (s, 3H)	1.27 (s, 3H)	1.30 (s, 3H)
19-H	1.29 (s, 3H)	–	–	1.27 (s, 3H)	1.30 (s, 3H)
20-H	–	3.61 (s, 3H)	–	–	–

[a]Refer structures (Table 1) for numbering.
S, singlet; d, doublet; t, triplet; m, multiplet; dd, double doublet.

Table 3. Comparative ^{13}C NMR for STS and the impurities identified

C[a]	STS (ppm)	Impurity I (ppm)	Impurity II (ppm)	Impurity III (ppm)	Impurity IV (ppm)
1-C	29.4	28.6	123.8	124.1	29.4
2-C	18.6	18.6	130.0	133.5	18.7
3-C	37.3	33.6	127.8	37.1	37.4
4-C	34.3	46.7	135.4	33.7	34.3
5-C	142.8	142.9	133.0	147.8	143
6-C	133.4	134.7	132.7	130.4	133.5
7-C	119.9	119.8	118.5	121.3	120.1
8-C	126.4	127.0	128.7	126.5	126.3
9-C	149.4	142.6	123.4	136	149.8
10-C	126.8	127.5	131.9	123.5	127
11-C	182.4	182.2	175.1	183.1	182.1
12-C	175.2	175.2	182.3	175.3	175.2
13-C	119.6	120.0	117.1	119.8	121.4
14-C	157.9	157.5	157.2	157.8	158.7
15-C	116.6	116.8	120.3	116.7	118.9
16-C	154.2	154.5	155.2	154.2	154.5
17-C	9.2	9.2	9.2	9.2	53
18-C	31.4	27.2	19.2	27.9	31.4
19-C	31.4	176.3	–	27.9	31.4
20-C	–	52.3	–	–	–

[a]Refer structures (Table 1) for numbering.

CONCLUSION

Six impurities in STS were observed and identified by HPLC. Impurity II (sodium tanshinone I sulfonate) is reported in literature, other five unknown impurities were isolated by separative HPLC and three parts were obtained (Impurity I, Impurity II, mixture of impurity III and impurity IV). They were characterised by LC-MS, UV, MS, and NMR techniques. Therein, impurity III and impurity IV need to be further separated and purified so as to get their respective single substance, and then be confired from the point of synthesis.

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Received December 3, 2008
Accepted April 14, 2009
Manuscript 6473