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Identification of potassium dehydroandrographolidi succinas and its major metabolites in rat urine by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A rapid, sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed for identification of potassium dehydroandrographolidi succinas and its metabolites in rat urine. Five male rats were administrated a single dose (100 mg/kg) of potassium dehydroandrographolidi succinas by *i.v.* injection. The urine were sampled from 0 to 24 h and purified by using Oasis® HLB extraction cartridge, then the purified urine samples were separated on a reversed-phase C18 column with a linear gradient and detected by an on-line MS detector. Identification and structural elucidation of the metabolites were performed by comparing their changes in molecular mass (Δm) and MS/MS spectra with those of the parent drug. Seven metabolites and the parent drug were found in rat urine. All these metabolites were reported for the first time.

1. Introduction

Potassium dehydroandrographolide succinate (DAS-K) is a derivative of andrographolide isolated from the leaves of *Andrographis paniculata*, which is traditionally used as a medicinal herb to treat different diseases in India, China and Southeast Asia [1,2]. Previous pharmaceutical studies and clinical application both suggested that DAS-K should have antibacterial and antiviral effects. It has been used widely in China for the treatment of virus pneumonia, malaria and respiratory infections [3–5]. Despite its important therapeutical values, its metabolism *in vivo* is not clear yet. Up till now, the studies only focused on determination of DAS-K and related substances in plasma and pharmaceutical formulations including spectrophotometry, HPLC and MS [6–9].

Recently, LC–MS/MS has been proved to be a powerful and reliable analytical approach for structural analysis of drug metabolites in biological matrices with high sensitivity and low consumption of samples [10–12]. In addition, MS/MS technique has made it possible to acquire rich structural information of interesting analytes. The metabolites can keep the basic structural features of parent drug after biotransformation. So the product ions of parent drug associated with their basic structural features obtained by MS/MS technique can be used as a substructural template of metabolite's analysis [13–16].

In this work, a sensitive and specific HPLC–MS/MS method was presented for identification of DAS-K and its metabolites in rat urine. The parent drug and its seven metabolites were firstly found in rat urine, which will be useful for future studies involving DAS-K, such as clinical therapy.

2. Materials and methods

2.1. Chemicals and reagents

DAS-K was kindly supplied by Henan Topfund Pharmaceutical Co. Ltd. (Zhumadian, China) with the purity >99%. Methanol (HPLC grade) was purchased from J. T. Baker, USA. Ammonium acetate and formic acid were from Sigma–Aldrich Co. Ltd. (Poole, UK). All of the aqueous solutions were prepared with water purified using a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

Stock solution of DAS-K was prepared by dissolving accurately weighed pure substance in methanol/water (50/50) to yield a concentration of 10 μg/mL.

2.2. Instrumentation and conditions

A waters HPLC system (Milford, MA, USA) including a 2695 separation module was controlled by Micromass Masslynx V4.1 SP4 software. Chromatographic separation was achieved on a Waters XTerra® MS C18 RP column (2.1 mm × 150 mm, 5 μm) with

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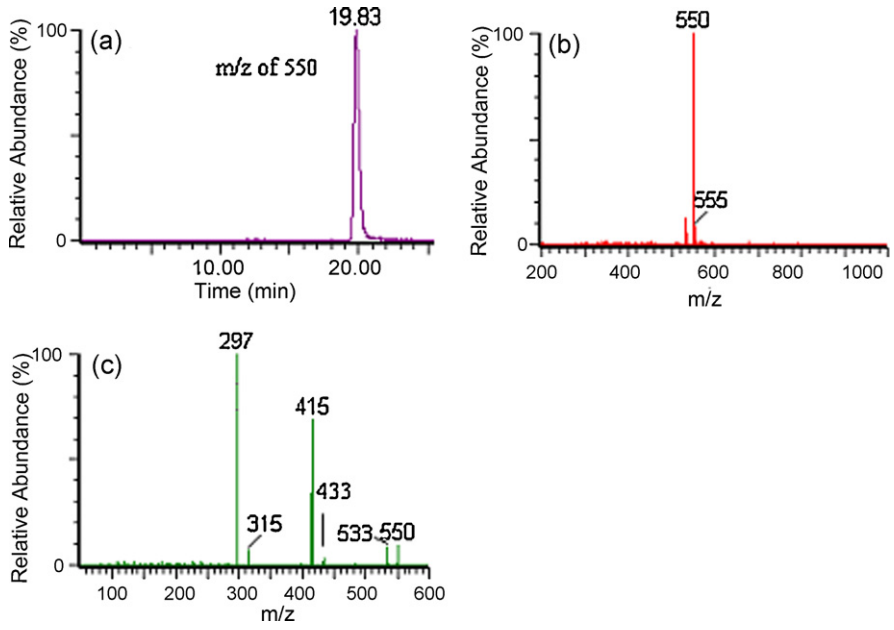

Fig. 1. The chromatogram and MS spectra of DAS-K. (a) Chromatogram; (b) MS spectrum; (c) MS/MS spectrum.

Waters C18 pre-column (2.1 mm × 10 mm, 5 μm) at a flow rate of 0.2 mL/min. The temperature of column was maintained at 30 °C. A gradient elution of B (methanol) and D (5.0 mM ammonium acetate, adjusted to pH 3.0 with formic acid) was used. The gradient was as follows: 20–60% B linear (0–5 min), 60% B linear (5–12 min), 60–80% B linear (12–20 min), 80–90% B (20–30 min). This was followed by a 10 min equilibrium period with initial conditions prior to injection of the next sample. Samples were filtered through 0.45 μm membrane and were subjected to HPLC–MS analysis with injection volume of 10 μL.

The HPLC system was online coupled to a Micromass Quattro-micro® triple quadrupole mass spectrometer equipped with ESI interface (Waters, Manchester, UK). Instrument control and data acquisition were performed using Micromass Masslynx V4.1 SP4 software. High purity nitrogen (N_2) was used as both drying gas and nebulizing gas, and ultrahigh pure argon (Ar) was used as the collision gas. HPLC conditions were the same as described above. Mass spectra were acquired in positive mode with a capillary voltage of 3.0 kV, a cone voltage of 19 V, a source temperature of 120 °C, a dry temperature of 300 °C, drying gas flow at 50 L/h, nebulizing

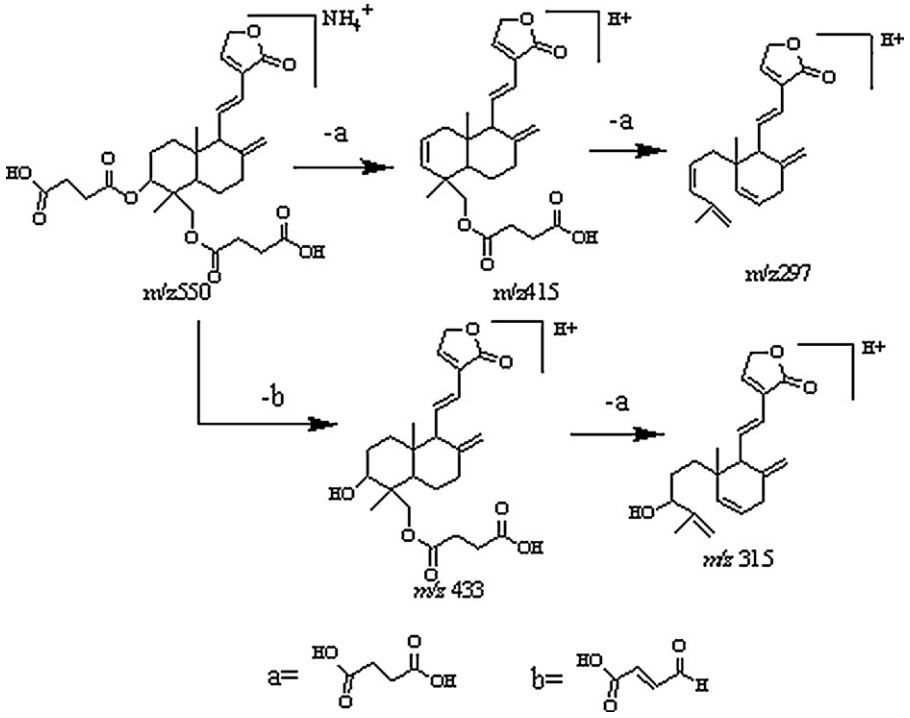

Fig. 2. The proposed fragmentation pathway of DAS-K.

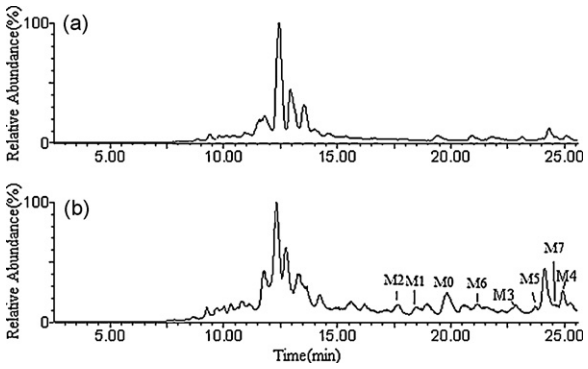

Fig. 3. Total ion chromatograms of SPE extract of (a) blank rat urine; (b) urine sample.

gas flow at 500 L/h, and collision induced dissociation (CID) was set at 18 eV.

2.3. Samples preparation

2.3.1. Administration

Five male Wister rats (200 ± 20 g, Henan Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine. All the animal studies were performed in the Specific Pathogen Free (SPF) laboratory. Animals used in these experiments were managed according to the rules and regulations of the Institutional Animal Care and Use Committee at Zhengzhou University, which also approved the experimental protocol. The rats were provided standard laboratory food and water *ad libitum*. The rats were fasted for 12 h with free access to water, then administered 100 mg/kg *i.v.* injection doses of DAS-K. Urine samples were collected for a period of 0–24 h and centrifuged at 3500 rpm for 10 min. The supernatant was collected and stored at −20 °C until analytes.

2.3.2. Urine extraction

Purification was performed with Oasis® HLB extraction cartridges (Waters, Milford, MA, USA) on a vacuum manifold (Waters, Milford, MA, USA) connected to an APOLP vacuum pump (Auto Science, China). Before use, the HLB cartridge was conditioned by adding sequentially 2 mL methanol and 2 mL water. Then one millilitre of mixed 0–24 h urine samples was loaded, and the HLB cartridge was washed with 1 mL water/methanol (90/10) to elute the impurity and 1 mL methanol to elute the analytes in turn. The methanol elution solutions were evaporated to dryness under nitrogen at 40 °C. The residues were dissolved in 200 μL of 50% methanol and 10 μL was injected for HPLC–MS/MS analysis.

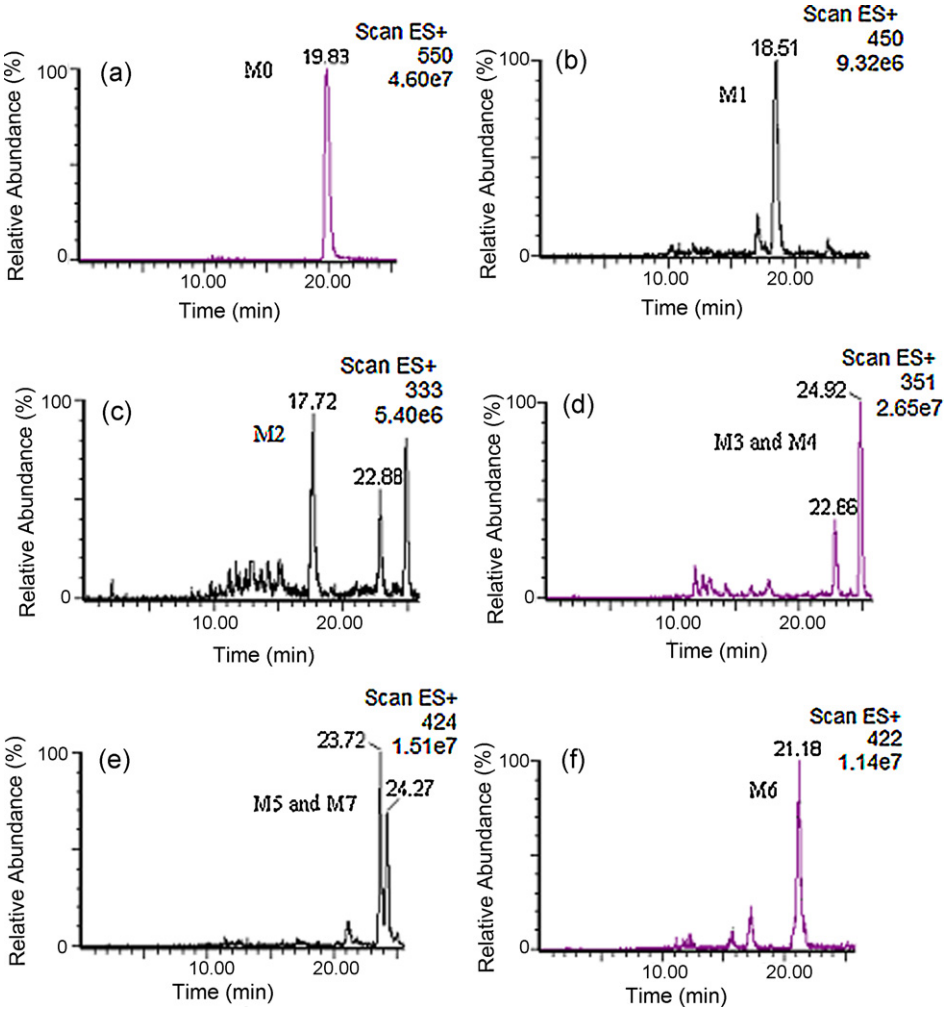

Fig. 4. Extracted ion chromatograms of DAS-K and its metabolites in rat urine.

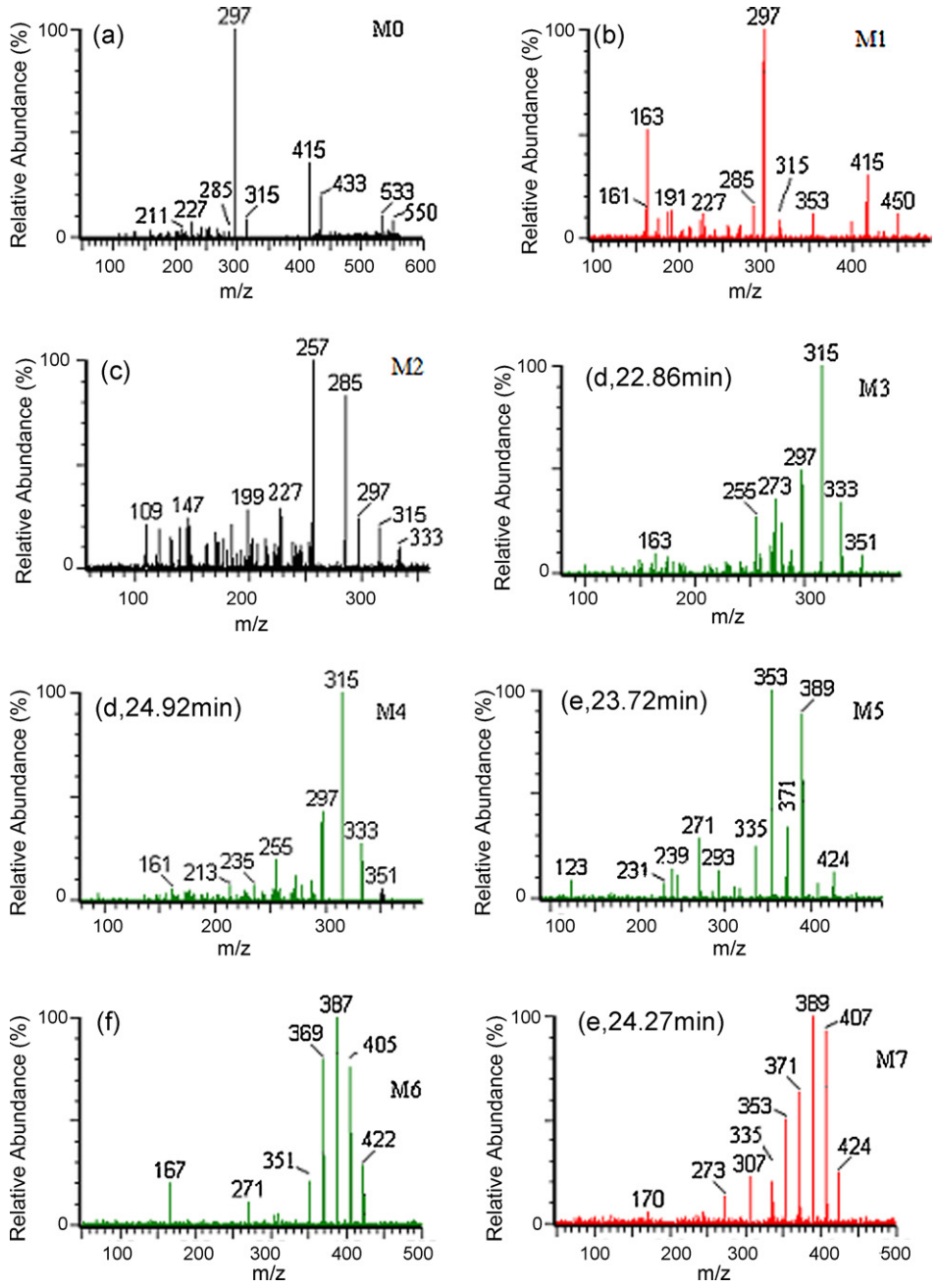

Fig. 5. The MS/MS spectra of DAS-K and its metabolites in rat urine.

3. Results and discussion

3.1. HPLC–MS and HPLC–MS/MS analysis of DAS-K

The HPLC–MS and MS/MS analyses of DAS-K were performed in positive ion detection mode. The chromatographic and mass spectrometric conditions were optimized using DAS-K standard. The HPLC–MS chromatogram and the MS/MS spectra of DAS-K were shown in Fig. 1. DAS-K was eluted after 19.83 min under above experimental conditions. DAS-K can form ammonium adduct molecular ion of *m*/*z* 550. The MS/MS spectrum showed an ion at *m*/*z* 533 corresponding to the loss of NH_3, an ion at *m*/*z* 433 corresponding to the loss of NH_3 and OHCCHCHCOOH (100 Da), an ion at *m*/*z* 415 corresponding to the loss of NH_3 and $HOOCCH_2CH_2COOH$ (118 Da), an ion at *m*/*z* 315 corresponding to the loss of NH_3, OHCCHCHCOOH (100 Da) and $HOOCCH_2CH_2COOH$ (118 Da), an ion at *m*/*z* 297 corresponding to the loss of NH_3 and two $HOOCCH_2CH_2COOH$ (118 Da). The product ions and the corresponding neutral fragment loss mentioned above were the characteristic structural information of DAS-K, and were the sound bases to identify metabolites of DAS-K. The mass fragmentation rule of DAS-K was shown Fig. 2.

3.2. HPLC–MS and HPLC–MS/MS analysis of metabolites

In order to identify the metabolites, the possible structures of metabolites were firstly speculated according to the rule of drug metabolism and the structure of parent drug. Then, total ion chromatogram of the purified rat urine after *i.v.* injection of DAS-K (Fig. 3b) was compared with those of blank rat urine (Fig. 3a) to find the possible metabolites. Finally, the possible metabolites were analyzed by HPLC–MS/MS to elucidate their structures through

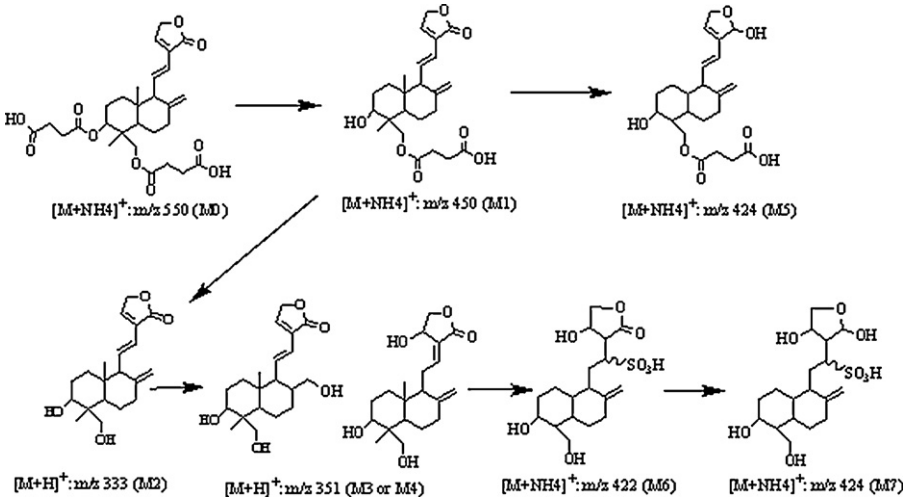

Fig. 6. The proposed *in vivo* metabolic pathways of DAS-K in rat.

comparing their retention times, changes in observed mass (Δm) and mass spectral patterns of product ions with those of DAS-K.

Based on the method mentioned above, DAS-K and its metabolites were found in rat urine after *i.v.* injection of DAS-K. Their molecular ions were at *m/z* 550, 450, 333, 351 (two), 422 and 424 (two), respectively. MS/MS spectra of the metabolites, obtained by CID of their molecular ions, were used for more precise structural identification of the metabolites. Extracted ion chromatograms of DAS-K and its metabolites were presented in Fig. 4. The MS/MS spectrum of each analyte was shown in Fig. 5. Among them, the retention time, the MS and MS/MS spectra of the molecular ion at *m/z* 550 (M0, Fig. 5a) were the same as those of DAS-K standard (Fig. 1c). Therefore, M0 can be the unchanged parent drug.

The ammonium adduct molecular ion of *m/z* 450 (M1, Tr = 18.51 min) was 100 Da less than the ammonium adduct molecular ion of DAS-K. The characteristic product ions at *m/z* 415, 315 and 297, and characteristic neutral loss 100 Da (OHCCHCHCOOH) of DAS-K were all appeared in the MS/MS spectrum of M1 (Fig. 5b), which were in close agreement with the fragmentation pathway of DAS-K. Thus, the molecular ion at *m/z* 450 was the metabolite of DAS-K resulted from the loss of one 4-oxobut-4-enoic acid. M1 can be identified as 14-deoxy-11,12-didehydro-andrographolide-19-succinate [17].

The protonated molecular ion of *m/z* 333 was 100 Da less than the ammonium adduct molecular ion of M1. Three chromatographic peaks giving rise to *m/z* 333 were detected in its extracted ion chromatogram (Fig. 4c), two of which were in close agreement with chromatographic peaks of *m/z* 351 (Fig. 4d). It was therefore deduced that two peaks at 22.88 and 24.92 min were the product ions of *m/z* 351. The MS/MS spectrum of M2 at 17.72 min yielded product ions at *m/z* 315, 297, 285 and 257 (Fig. 5c). *m/z* 315 and 297 corresponded to the characteristic fragment ions of DAS-K. Thus, the molecular ion at *m/z* 333 was the product of the loss of one 4-oxobut-4-enoic acid from M1. M2 can be identified as dehydrographolide [18].

The protonated molecular ion of *m/z* 351 was 18 Da more than the ammonium adduct molecular ion of M2. Two chromatographic peaks giving rise to *m/z* 351 were detected in its extracted ion chromatogram (Fig. 4d), the retention times were 22.86 min (M3) and 24.92 min (M4). The characteristic product ions at *m/z* 315 and 297 of DAS-K were also appeared in the MS/MS spectrum of *m/z* 351 (Fig. 5d). Thus, the molecular ion at *m/z* 351 was the product of adding of water across a double bond in M2. M3 or M4 can be identified as andrographolide [18]. In addition, one of the hydroxylations to yield M3 or M4 probably occurred on the methylene group on the cyclohexane ring of M2.

The ammonium adduct molecular ion of *m/z* 424 (M5, Tr = 23.72 min) and its daughter ions at *m/z* 389, 371 and 271 (Fig. 5e) were all (28-2) Da less than the ammonium adduct molecular ion of M1 and its daughter ions at *m/z* 415, 397 and 297, respectively. The fragment ion at *m/z* 271 was formed by loss of 118 Da ($HOOCCH_2CH_2COOH$) from the ion at *m/z* 389. The fragment ion at *m/z* 335 was formed by loss of 72 Da ($CH_2{=}CH_2COOH$) from the ion at *m/z* 407. Thus, M5 can be the simultaneously dedimethyl and di-hydrogenating product of M1. According to the result of Thevis [19], we inferred that the locations of de-methyl could be at C-4 and C-10, and the hydrogenating reaction occurred on the –C=O group on the lactonic ring of M1, respectively.

The ammonium adduct molecular ion of *m/z* 422 (M6, Fig. 5f) and its daughter ions at *m/z* 387, 369 and 351 were all increased by (80 + 2–28) Da when compared with that of the protonated molecular ion of andrographolide and its daughter ions at *m/z* 333, 315 and 297. The fragment ion at *m/z* 387 was formed by the loss of 18 (H_2O) from the *m/z* 405. The loss of 18 (H_2O) from the *m/z* 387 produced the ion at 369. The further loss of 18 (H_2O) from the *m/z* 369 produced the ion at 351. The loss of 80 (SO_3) from the *m/z* 351 produced the ion at *m/z* 271. Based on these data, M6 can be identified as the simultaneously dedimethyl and sulfonate product of andrographolide. According to the results of Yao [20,21] and Thevis [19], we inferred that the sulfonate conjugation could be located at C-12 in andrographolide, and the locations of de-methyl could be at C-4 and C-10, respectively.

The ammonium adduct molecular ion of *m/z* 424 (M7, Tr = 24.27 min) and its daughter ions at *m/z* 407, 389, 371 and 353 (Fig. 5e) were all increased by 2 Da when compared with that of the ammonium adduct molecular ion of M6 and its daughter ions at *m/z* 405, 387, 369 and 351. The MS/MS spectra of *m/z* 424 → 407 → 389 → 371 → 353 were the same as the MS/MS spectra of M6. The fragment ion at *m/z* 335 was formed by the loss of 18 (H_2O) from the *m/z* 353. The loss of 80 (SO_3) from the *m/z* 353 produced the ion at *m/z* 273. Thus, M7 can be the hydrogenating product of M6, and the hydrogenating reaction occurred on the –C=O group on the lactonic ring of M6.

Based on the above discussion, the proposed major metabolic pathway of DAS-K *in vivo* was shown in Fig. 6.

4. Conclusions

For the first time, the metabolites of DAS-K in rat urine were analyzed by the presented method. DAS-K and its major metabolites were identified through comparing the changes in observed mass (Δm) and tandem MS spectra with those of the parent drug. The results indicated that there were seven metabolites in rat urine, such as loss of 4-oxobut-4-enoic, de-methyl, hydrogenation, hydroxylation and sulfonate conjugated metabolites and so on. All these metabolites were reported at the first time.

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