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Short communication

Determination of scutellarin in *Erigeron breviscapus* extract by liquid chromatography–tandem mass spectrometry

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Abstract

A quantitative assay for scutellarin by LC–MS–MS (negative ion mode) was developed. The scutellarin was extracted from dry *Erigeron breviscapus*. Significant ion suppression was observed, which could be eliminated by increasing the turboionspray interface temperature to 350°C and by 1000-fold dilution of the extract with solvent. The calibration curve of scutellarin showed excellent linearity over a wide concentration range (0.01–100 μg/ml) (r=0.998), and the limit of detection was 15 pg/ml using a 10-μl injection volume. The analysis time was 4 min/sample.

Keywords: *Erigeron breviscapus*; Scutellarin

1. Introduction

Scutellarin, an effective constituent of several species of plants such as *Erigeron breviscapus*, can pass through the blood–brain barrier and prevent agglutination of the blood platelets [1,2]. Scutellarin is currently under development as a natural drug for the treatment of cerebrovascular diseases such as hemiplegia. There has been no report on the accurate quantitation of scutellarin until now, except Ref. [3] which described a semiquantitative assay by thin-layer chromatography (TLC) or UV detection. This is due to the difficulty [3] of separating scutellarin from other flavones in natural products by routine liquid chromatography (LC) or UV methods.

The technique of LC–tandem mass spectrometry (MS–MS) [4–9] is well suited to mixture analysis and it has been regarded as an ideal method that allows the analyst to develop highly sensitive and rapid assays of complicated mixture [10,11]. Unfortunately, the reliability of quantitative assays may not be absolute when the sample matrix is too complex [12–16]. It has been reported [12] that the matrix effect could be minimized by employing selective extraction and increasing high-performance liquid chromatographic (HPLC) separation.

In this paper, a rapid method for the determination of scutellarin in *Erigeron breviscapus* extract was described, and the severe ion suppression effect observed during the development of this method was eliminated. To our knowledge, this is the first

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method for the accurate determination of scutellarin, and the first report for minimizing ion suppression effect under the LC conditions of low retention.

2. Experimental

2.1. Chemicals and equipment

Scutellarin was from the Delta Information Center for Natural Organic Compounds (99.5%, Hong Kong). HPLC-grade methanol and acetonitrile was from Fisher (Hong Kong). Dry *Erigeron breviscapus* plant was obtained from the Yun Nan province, China.

A PE Sciex (Thornhill, Canada) API 3000 triple-quadrupole tandem mass spectrometer equipped with a turboionspray interface, an online degasser and a Perkin-Elmer binary pump (Model 250) was used for LC–MS–MS. The data were processed using Macquan software (PE Sciex).

2.2. Sample preparation

2.2.1. Extraction

Dry *Erigeron breviscapus* was ground to fine powder by a pulverizer. A 0.2-g amount of the fine powder was placed in a 50-ml capped stainless steel vessel and 10 ml methanol was added. The vessel was closed and the extraction was carried out in an ultrasonic washer. The temperature ranged from 35 to 42°C after 10 min extraction. The solution was centrifuged for another 10 min at 8000 rpm, and the supernatant was transferred to a beaker. The extraction procedure was repeated five times, when no scutellarin was detected in the supernate by LC–MS–MS. All the supernates were combined.

2.2.2. Spike after extraction

When the effect of interface temperature and sample concentration on the ion suppression effect was investigated, the extract instead of the plant powder was spiked with standard solutions and assayed to measure the extent of the ion suppression effect.

2.2.3. Dilution

A 500-μl volume of the (spiked) extract was transferred to different volumetric flasks in aliquots, and 10-fold, 100-fold, or 1000-fold diluted with methanol.

2.3. LC–MS–MS

The LC eluent was water–acetonitrile (20:80) and the analytical column was a Megachem C_{18} column (50 mm×2.1 mm I.D., 5 μm particle size). The flow-rate was 0.2 ml/min and the sample volume was 10 μl. The LC effluent was split 1:4 using a post-column split. The negative-ion mode of MS–MS was adopted and multiple reaction monitoring (MRM) for tandem MS was applied. The mass spectrometer was programmed to monitor the deprotonated molecule $[M-H]^-$ at m/z 461 via the first quadrupole filter (Q_1), and collision-activated dissociation (CAD) was performed (collision gas N_2, 4 p.s.i., collision energy 28 eV) at Q_2 (1 p.s.i.= 6894.76 Pa). The product ion, m/z 285, was monitored via Q_3. The ionspray voltage, orifice potential and ring focus voltage were set at −3400 V, −46 V and −160 V, respectively. The dwell time was 400 ms. The flow-rates of nebulizer gas (air), curtain gas (nitrogen) and drying gas (nitrogen) were, respectively 10 ml/min, 12 ml/min, 1.2 l/min. In order to reduce the matrix effect, a high interface temperature of 350°C (see below) was used. Under the above conditions, the ion source was thermally stabilized for 30 min before injection. Peak areas obtained from the MRM of scutellarin were utilized for the construction of a calibration curve.

3. Results and discussion

3.1. LC–MS–MS optimization

Since the glucosidic bond of scutellarin is easily cleaved to generate a negative product ion at m/z 285 in the collision cell, we can obtain good selectivity by using the negative-ion mode. The optimization was carried out in a three-step process.

First, Q_1 (−) scan mode was used to investigate the $[M-H]^-$ ion (at m/z 461) of scutellarin (shown in Fig. 1A). Secondly, we used product ion scan to look for the most abundant product ion of $[M-H]^-$(shown in Fig. 1B). Among the product ions, the

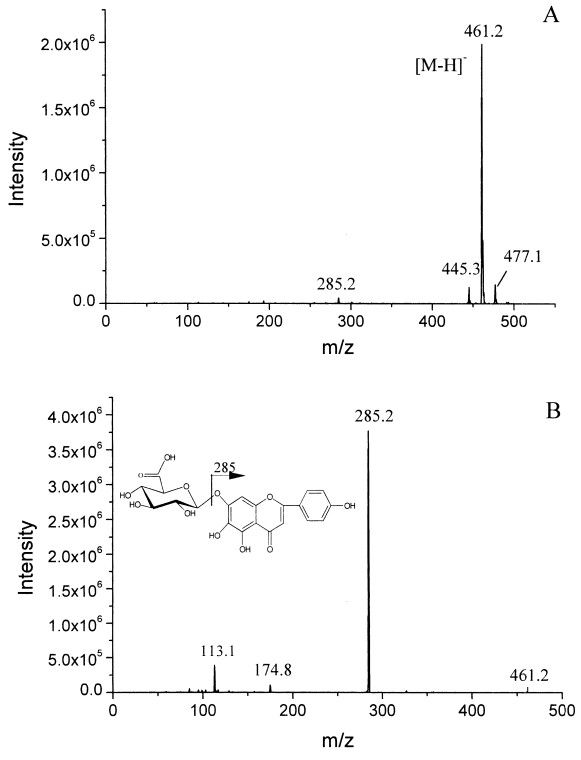

Fig. 1. (A) Negative-ion Q_1 mass spectrum of scutellarin; (B) full-scan product ion spectrum of scutellarin.

ion at m/z 285 was the most abundant, therefore the precursor/product ion pair of m/z 461/285 was chosen for the MRM scan. Thirdly, we sampled through the LC column and optimized the LC conditions.

The retention time of scutellarin was 2.8 min (Fig. 2).

3.2. Attempted assay validation

We attempted to validate the assay in the concentration range of 0.2–50 μg/ml with undiluted extract and an interface temperature of 100°C, when a significant ion suppression effect was found. Although the linearity of the calibration curve was good (0.993), the relative standard deviations (RSDs) and recoveries were highly unsatisfactory (40–60% and 51–65%, respectively). We repeated the same validation experiment except that the scutellarin was spiked to the extract *after* extraction, and the data obtained were comparable to that of

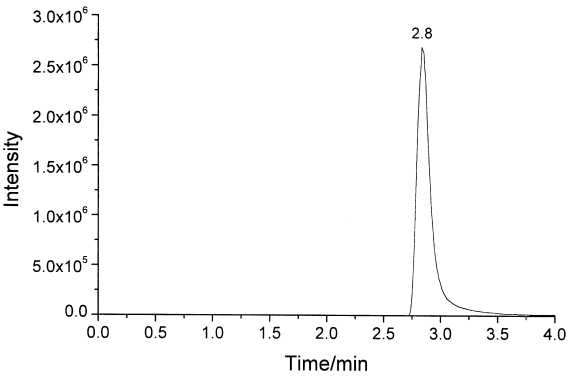

Fig. 2. MRM chromatogram of a standard solution containing 2 μg/ml scutellarin.

before extraction. We also repeated the validation experiment but spiked standards to 30 μg/ml scutellarin (close to the concentration in the extract) *neat* methanol solution instead of the extract, and found that the precision was better than 7% and the recovery values ranged from 94 to 102%. Therefore, it can be deduced that the complex matrix of the extract rather than the inefficiency and variability of the extraction efficiency was responsible for the poor precision and recovery of the assay.

3.3. The definition of ion-suppression%

It had been concluded from the results of above experiments that the poor recovery was caused by the difference of ion responses among the standards and sample matrix, not by poor extraction efficiency. Therefore, we employed ion-suppression percent as an indication of this ion suppression effect. We defined the ion-suppression% as:

$$\text{Ion-suppression\%} \equiv 1 - \frac{\text{added amount found by LC–MS–MS}}{\text{amount added}} \cdot 100\%$$

(the extract was spiked *after* extraction).

The value of (1 − ion-suppression%) is actually the ratio of the MS response of an analyte in a sample matrix to that in standard solutions. An ion-suppression% approximate to 0% is desirable for the LC–MS–MS external standard method, as it means

that the analyte has basically the same MS signal response among standard solutions and samples.

3.4. The influence of interface temperatures and dilution ratios of the extract on the precision and ion-suppression%

Since the interface temperature has a great effect on the ionization efficiency, we first studied the relationship between interface temperature and the ion suppression effect. We spiked the extract *after* extraction at five levels (0.1–50 μg/ml) and assessed the ion-suppression% and precision at various interface temperatures. The ion-suppression% values were essentially the same at 100, 200 and 350°C, while the precision of the data (RSD) increased remarkably, from 35–60 via 20–30 to 7–12%. However, it is obvious that the ion-suppression% was still too high (>31%) to be accepted for practical use.

In our experiment, we found that when more solvent was used for extraction, the ion-suppression% decreased notably. This opened the possibility that the ion suppression effect can be effectively minimized by dilution of the sample. We evaluated the effect of dilution of the extract on the ion suppression effect by diluting the spiked extract with methanol and assaying the ion-suppression% and precision experimentally at the interface temperature of 350°C. The precision of the data at dilution ratios of 10-fold, 100-fold and 1000-fold ranged from 6 to 11%, while the ion-suppression% decreased dramatically from 20–30 via 10–15 to 1–5%. Although the molar ratios of the analyte to other compounds in the matrix of the extract did not change after dilution, the ion suppression effect caused by those compounds was virtually alleviated. We deduced from this phenomenon that the interaction between the analyte molecule and those of other compounds in the extract matrix was greatly weakened after dilution, and consequently, the ion suppression effect was remarkably reduced.

3.5. Linearity and limit of detection

Standard solutions were prepared in methanol at concentrations of 0.01–100 μg/ml. The regression equation (peak-area, A, vs. concentration, C, in μg/ml) and its correlation coefficient was calculated as follows:

$$A = 7.94 \cdot 10^6 C - 57.5 \; (r = 0.998)$$

The estimated detection limit – the signal projected to give a signal-to-noise ratio (S/N) of 3:1 – corresponded to concentration of 15 pg/ml.

Table 1
The average recoveries and RSDs for the determination of scutellarin under optimized conditions ($n=6$)

	Fortified level (μg/ml)				
	0.1	0.5	2.0	10	50
Precision (RSD, %)	7.8	5.1	6.4	7.7	9.2
Recovery (%)	91	88	89	95	93

3.6. Recovery, precision and calculated content

The recoveries of scutellarin from dry plant powder and the RSDs are summarized in Table 1. These results are based on 30 recovery determinations in dry plant powder spiked before extraction.

It is calculated from the result of the determination that scutellarin accounted for 0.56% (w/w) of the dry *Erigeron breviscapus*.

4. Conclusions

The high selectivity of MRM detection enables rapid analysis of active compounds in their plant sources without the necessity for a HPLC baseline separation. However, a significant ion suppression effect was often observed in such analysis. We found that both the source temperature and the dilution ratio of the extract had great effects on the data precision and ion-suppression% values of the analyte. It is proved that the ion suppression effect could be minimized and a satisfying result obtained by adopting a high interface temperature and 1000-fold dilution of the extract.

This method has already demonstrated good recoveries, good precision and sensitivity. The analysis time was only 4 min/sample. However, the dilution of sample is disadvantageous when the content of an analyte in a sample is too low.

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