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Journal of Chromatography A, 928 (2001) 155–162

JOURNAL OF CHROMATOGRAPHY A

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Identification and determination of glucuronides and their aglycones in *Erigeron breviscapus* by liquid chromatography–tandem mass spectrometry

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Received 25 April 2001; received in revised form 5 July 2001; accepted 11 July 2001

Abstract

A novel, speedy and reliable LC–MS–MS method for the search, identification and quantitation of *O*-glycosides and their aglycones from plant resources was established by analyzing the extract of *Erigeron breviscapus*. The extract was directly infused to a triple–quadrupole MS–MS and major glucuronides in the extract were screened out with high confidence by a neutral loss scan for the loss of a gluconic acid. The identity of these glucuronides and their aglycones was further confirmed with LC–MS–MS. In addition to scutellarin, apigenin 7-*O*-glucuronide, quercetin-3-*O*-glucuronide and their aglycones that were previously reported by others, we also confirmed by LC–MS–MS that remarkable amount of baicalin, an isomer of apigenin 7-*O*-glucuronide, presented in the extract of this plant, which had not been reported before. A satisfying quantitation of three glucuronides was also made by LC–MS–MS.

Keywords: *Erigeron breviscapus*; Flavonoids; Glucuronides; Glycosides

1. Introduction

Glycosides are important phytochemicals. They are metabolized in vivo from flavones, anthraquinone, lignanoids, etc., and many of them are biologically active [1]. Some specific glycosides had been analyzed in biological matrices by colorimetric methods [2], thin-layer chromatography (TLC) [3], column liquid chromatography (LC) [4–8], capillary electrophoresis (CE) [9], gas chromatography–mass spectrometry (GC–MS) [10,11], MALDI-TOF MS [12], LC–MS [13,14], and LC–MS–MS [15]. However, until now there has been no method that can be employed to seek the glycosides (known or unknown) out in biological samples.

Due to its advantage in the analysis of complex mixtures, LC–MS–MS is a very powerful tool for bioanalysis [16]. The scan modes of product scan, selected reactions monitoring, neutral loss scan and precursor scan of MS–MS enable rapid acquisition of useful structural information of the unknown ingredients from a complex matrix, yet it is surprising that the use of the later two scan modes was rarely reported. In this paper, we established a method for the analysis of *O*-glycosides from a biological matrix: a screening of the glycosides by neutral loss scans with MS–MS, followed by the identification of the glycosides and their aglycones

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)01111-6

by LC–MS–MS. This method was set up by analyzing the glucuronides and their aglycones in an extract of *Erigeron breviscapus*.

Erigeron breviscapus has been used as a medicinal herb for the treatment of paralysis caused by cerebral vascular accidents such as hypertension, cerebral embolism, and chronic arachnoiditis [17]. It has been proved that its major active compounds were glucuronides [17]. The ingredients of *Erigeron breviscapus* had been studied with UV, HPLC and NMR [18,19]. The plant used for this study was *Erigeron breviscapus* (Vant.) Hand.–Mazz from southern China, whose constituents had not been sufficiently studied. The glucuronides and their aglycones involved in this report are shown in Table 1.

This study reports the first method specifically for the search and confirmation of *O*-glycosides and their aglycones from a biological matrix. LC–MS–MS was also used for structural elucidation and quantitation of the glycosides.

2. Experimental

2.1. Chemicals and equipments

Scutellarin and scutellarein were from Delta Information Center for Natural Organic Compounds (99.5%, Hong Kong, China). Apigenin, baicalin, and quercetin were purchased from Fluka (Buchs, Switzerland). Apigenin 7-*O*-glucuronide and quercetin-3-*O*-glucuronide were kindly contributed by the Yunnan Institute of Phytochemistry (99%, Kun Ming, China). HPLC-grade methanol and acetonitrile were from Fisher (Hong Kong, China). Dry plant of *Erigeron breviscapus* (vant.) Hand.–Mazz was obtained from Yun Nan province, China.

A PE SCIEX (Toronto, Canada) API 3000 triple–quadrupole tandem mass spectrometer equipped with a turbo ion spray Interface, an online degasser and a Perkin-Elmer binary pump (model 250) were used for LC–MS–MS analysis. The data were processed using Macquan software (PE Sciex).

2.2. Sample preparation

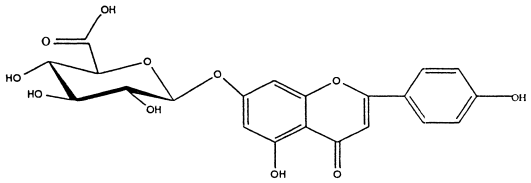
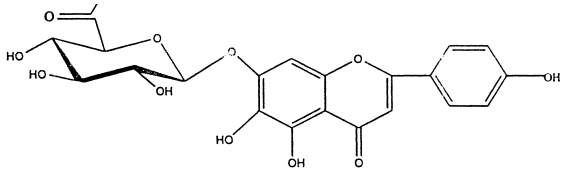
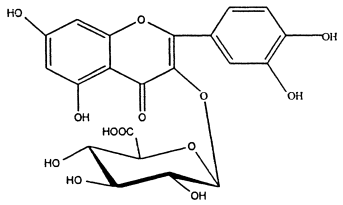
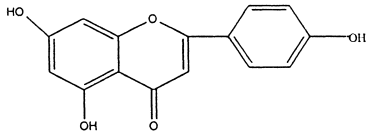
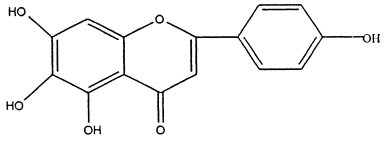
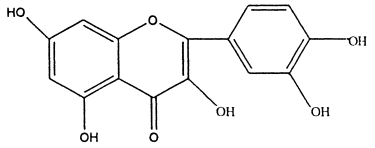
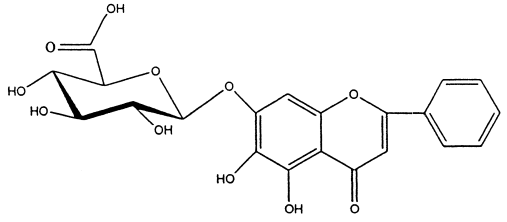
The dry plant of *Erigeron breviscapus* was ground to fine powder by a pulverizer, and 0.2 g of the fine powder was placed in a 50-ml capped stainless steel vessel and 10 ml methanol were added. The vessel was closed and the extraction was carried out in an ultrasonic washer. The temperature ranged from 35 to 42°C after 10 min extraction. Then the solution was centrifuged for another 10 min at 8000 rpm, and the supernatant was transferred to a beaker. The above extraction procedure was repeated for five times. All the supernates were combined.

2.3. Qualitative LC–MS–MS

Neutral loss scan of MS–MS, selected-ion monitoring and product scan of LC–MS–MS were employed for the qualitative analysis. For the neutral loss scan, the extract was directly infused to the MS–MS by a flow syringe pump at 0.3 ml/h. Negative-ion mode of MS–MS was adopted. The MS parameters including collision energy and spraying conditions were optimized with standard of luteolin-7-glucuronide. The mass spectrometer was programmed to scan for the deprotonated molecules that had a neutral loss of 176 after collision-activated dissociation (CAD) at the collision cell (Q_2, collision gas N_2, 4 p.s.i, collision energy 24 eV) over the m/z range from 200 to 800. The ion spray voltage, orifice potential and ring focus voltage were set at −3400, −46 and −160 V, respectively. 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. To avoid carryovers among samples, the system was rinsed at a flow-rate of 8 ml/h according to the following order: 100% methanol (5 min), thin aqueous ammonia (pH 9.5, 5 min), water (5 min) and methanol (10 min) between two successive assays.

As for the identification of the compounds by LC–MS–MS, separations were carried out on a Luna C_{18} column (250×4.6 mm I.D., 5 μm, Phenomenex, Torrance, CA, USA) operated at 45°C, using a mobile phase consisting of acetonitrile–water at a flow-rate of 1 ml/min. The proportion of acetonitrile was 10% (v/v) for the first 10 min of the run, and then increased linearly to reach 40% (v/v) at 45 min. The sampling volume was 20 μl and the LC effluent was split 1:10 using a post-column split. Selected ion monitoring (SIM), selected reaction monitoring (SRM) and product scan were employed for the

Table 1
Structure of compounds involved in this study

Compound	M.W.	Structure
Apigenin 7-*O*-glucuronide (I)	446	
Scutellarin (II)	462	
Quercetin-3-*O*-glucuronide (III)	478	
Apigenin (IV)	270	
Scutellarein (V)	286	
Quercetin (VI)	302	
Baicalin (VII)	446	

further confirmation of the glucuronides and their aglycones in the extract. Six ions were monitored simultaneously and the dwell time for each ion was 100 ms for the SIM mode. For the product scan, the spectra were accumulated 10 times. Other MS conditions were the same as those of the neutral loss analysis, except that temperature of the ion source was maintained at 250°C and no CAD gas was introduced when SIM mode was employed.

2.4. Quantitative LC–MS–MS

The analytical column for the quantitative LC–MS–MS was a Megachem C_{18} column (150×2.1 mm I.D., 5-μm particle size) operated at 45°C. Solvent A was water and solvent B was acetonitrile. Gradient conditions: 0–5 min 10% B; 5–25 min 10–50% B; the flow-rate was 0.2 ml/min. The extract was 100-fold diluted before injection and the sampling volume was 20 μl. Glucuronides were detected by tandem mass spectrometry using selected reaction monitoring (SRM) of the transitions *m/z* 477→371, *m/z* 461→285 and *m/z* 445→269. The collision energies for those transitions were, respectively, 23, 19 and 17 eV. The dwell time for each transition was 200 ms. The interface temperature was set to 350°C and the ion source was thermally stabilized for 30 min before injection. Other MS conditions were the same as those of the qualitative method.

2.5. Quantitative determination/recovery measurement and validation procedures

The external standard method of calibration was used for this method. Standard solutions of glucuronides were prepared in methanol at the concentrations of 0.01, 0.05, 0.2, 0.5, 1.0, 2.0, 5.0, 10 μg/ml. Calibration curves were prepared by plotting the average peak areas of extracting ion currents (XICs) versus the compound's concentrations. Determination of glucuronides in the extract was done using weighted least-squares regression analysis of the standard curves.

Recovery of this method was determined by standard additions to the dry plant powder at five levels (from 0.05 to 10 μg/ml); then the powder was volatilized to dryness and the added amounts experimentally measured.

3. Results and discussion

3.1. Qualitative analysis

3.1.1. Neutral loss scans

Under negative ion mode, the glucosidic bond of *O*-glucuronides is easily cleaved in the collision cell to generate product ions of $[M–H–176]^-$, which correspond to the fragments resulted from the neutral loss of a gluconic acid from the deprotonated molecule. This opens the possibility that the unknown *O*-glucuronides within a certain *m/z* range could be sought out from a biological sample by employing a neutral loss scan for the *m/z* loss of 176 over that range.

The neutral loss scan spectrum of the extract of *Erigeron breviscapus* (Vant.) Hand.–Mazz is shown in Fig. 1. Three notable peaks, respectively, at *m/z* 445, 461 and 477 were found within the *m/z* range from 200 to 800 (there was no apparent peak observed after *m/z* 800), while none of these peaks was visible on the rather complicated Q1 scan spectrum over this range (not shown). Referring to the forgone information on the chemical composition of this plant and its congeneric and conspecific plants, we presumed that these three ions were

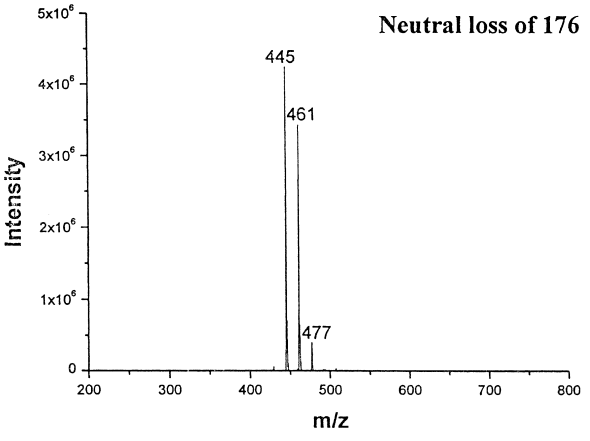


Fig. 1. The spectrum of the neutral loss scan for176 of the extract of *Erigeron breviscapus* (Vant.) Hand.–Mazz.

apigenin 7-*O*-glucuronide, scutellarin and quercetin-3-*O*-glucuronide, respectively. This needed further confirmation by LC–MS–MS.

3.1.2. Qualitative LC–MS–MS

Since flavones usually have isomers, sufficient HPLC separation is necessary for the LC–MS–MS qualitative analysis of the extract. Therefore, we employed a relatively slow gradient for the HPLC. The SIM chromatograms are shown in Fig. 2. Besides the ions of the three glucuronides, respectively, at m/z 445, 461, and 477, we also monitored the ions of their aglycones at m/z 269, 285 and 301 simultaneously with LC–MS. Confirmation of compounsd I, II and III labeled in Fig. 2A–C was made by using their standard solutions, which showed peaks at the same retention times. On the SIM chromatograms of the aglycones (Fig. 2D–F), peaks that had the same retention times as the glucuronides corresponded to the in-source-decomposition fragments of the deprotonated molecule of those glucuronides. The peaks of compounds IV, V and VI were identified by comparing the retention times against those standards.

3.1.3. Confirmation of baicalin

A notable peak other than that of apigenin 7-*O*-glucuronide (compound I) on the SIM chromatogram of m/z 445 was found at the retention time of 31.1 min (Fig. 2A). The peak also appeared in the selected reaction monitoring (SRM) chromatogram of m/z 445→269 (LC–MS–MS chromatogram not shown). Therefore, it is likely that this peak was from another glucuronide, which was an isomer of compound I. We investigated the product ions of m/z 445 with LC–MS–MS (shown in Fig. 3), and found that the product spectrum of the unknown compound and compound I were analogous, except that the unknown compound did not have a product ion at m/z 191 (Fig. 3B). The product spectrum of the unknown compound was compared against that of serveral possible glucuronides (including the glucosdiurionides of rhabarberone, galangin, baicalein and norwogonin), and was found congruent with that of baicalin, the glucosdiurionide of baicalein. The product of compound I at m/z 191 was generated by a hexatomic rearrangement (Fig. 3A), while the 6-OH group of baicalin makes this reaction unlikely because of its steric hindrance and electric effect. In addition, we found that the unknown compound had the same retention time as bacailin.

From the information of its molecular mass, product ions and retention time, we can confirm that the unknown compound was baicalin. This compound has not been reported in this genus of plant before.

3.2. Quantitative analysis

Deprotonated glucuronide is liable to generate an abundant product ion of $[M–H–176]^-$ in the collision cell. Therefore, we selected the precursor→product transitions of $[M–H]^-$→$[M–H–176]^-$ for the SRM detection of compounds I, II and III to obtain good sensitivity and selectivity.

The regression equations, linearities, detection limits and retention times of compounds I, II and III are shown in Table 2. All of the calibration curves shown good linearity (0.992–0.997) over the concentration range from 0.01 to 10 μg/ml.

For the LC–MS–MS quantitation of biological samples, the accuracy of LC–MS–MS determination was often impaired by ion suppression effect, which is caused by sample matrix [20–23]. In recent work [24], we investigated the matrix effect occurring in the quantitation by LC–MS–MS, and proved that a reliable quantitative assay with a triple–quadrupole MS using an external method can be achieved, if several measures are taken. In this study, since we had employed a high source temperature and high HPLC separation, besides that the extract was 100-fold diluted, no obvious ion suppression effect was observed. This found expression in the good recoveries and precisions of the determinations (shown in Table 3). The C.V. of compound III was higher (14%) than the other two compounds. The authors believed that it was caused by relatively severe in-source decay of its deprotonated molecule.

The results of the quantitation are also shown in Table 3.

4. Conclusions

A new MS–MS and LC–MS–MS method for the screening, confirmation and quantitation of glyco-

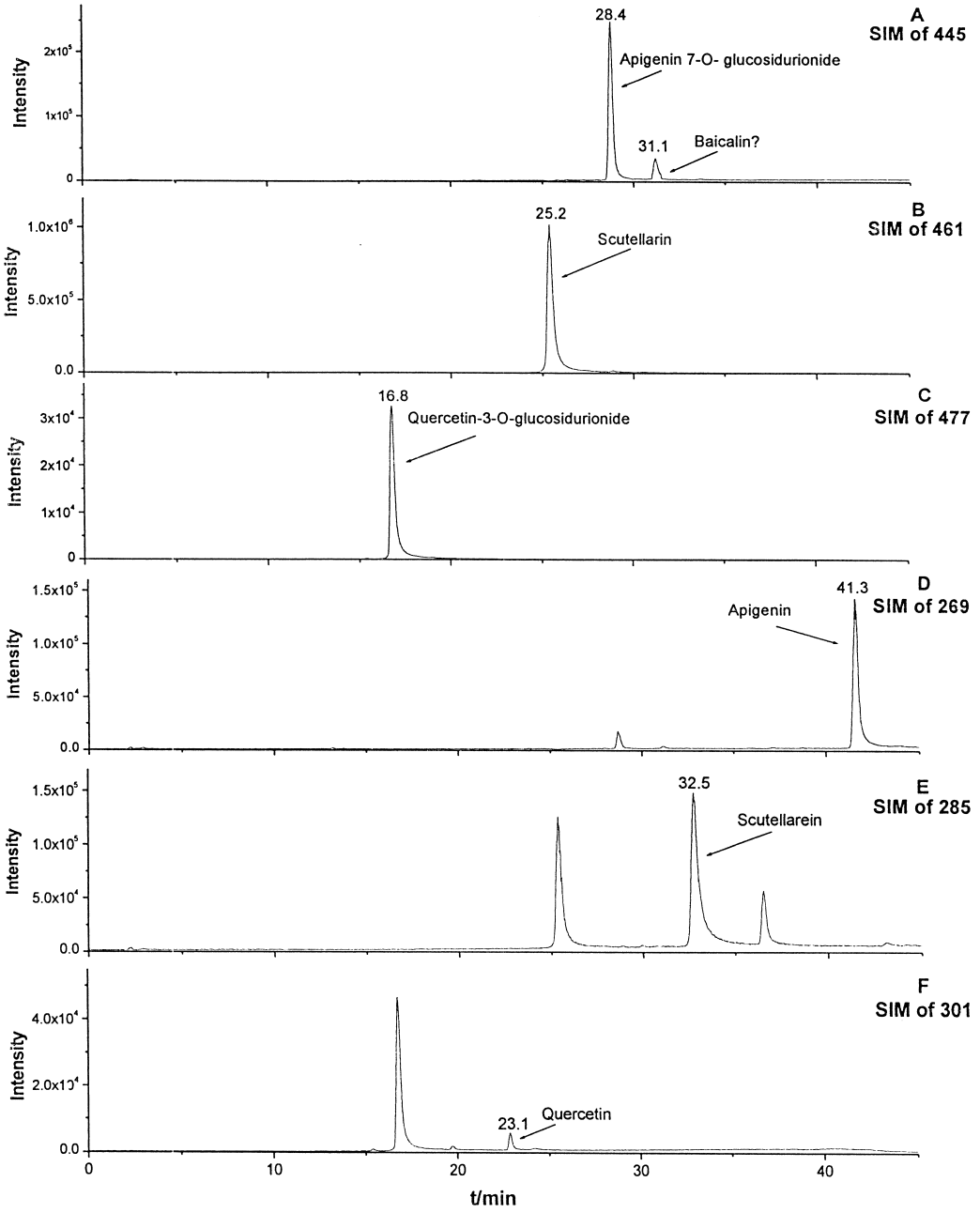


Fig. 2. Identification of the glucuronides and their aglycones in the extract of *Erigeron breviscapus* (Vant.) Hand.–Mazz by single-ion monitoring (SIM).

sides and their aglycones in plants has been illustrated with the extract of *Erigeron breviscapus*. This method started with screening of glycosides by neutral loss scans with MS–MS for the neutral loss of certain sugars, followed by confirmation and quantitation with LC–MS–MS.

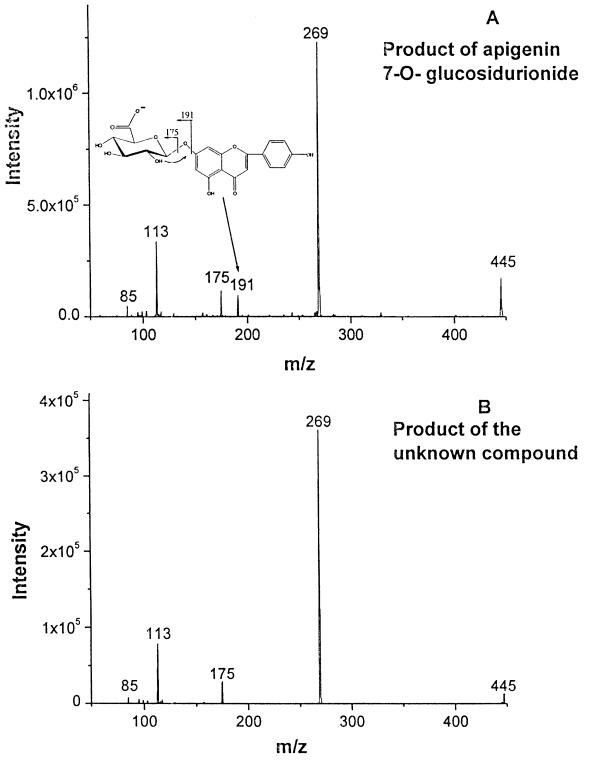


Fig. 3. The spectra of product ions of m/z 445 in the extract of *Erigeron breviscapus* (Vant.) Hand.–Mazz by LC–MS–MS; (A) the accumulated product ions spectra from 28.2 to 28.6 min (compound I); (B) the accumulated product ions spectra from 29.9 to 31.3 min (unknown compound).

Without the need of phytochemical separation or much sample preparation, the glucuronides and their aglycones in the extract were sought out and identified with high selectivity, sensitivity and confidence. In addition, the presence of a notable amount of baicalin in this plant was confirmed, which has not been reported before. The quantitation of the three glucuronides was not affected by ion suppression effect because high source temperature, high HPLC separation and dilution of the sample were adopted.

Table 3
Quantitation of glucuronides in the extract by LC–MS–MS ($n=6$)

Compound	Mean (mg/g)	%C.V.	Average % recovery
I	14.3	8.9	86
II	5.8	6.7	91
III	0.83	14	87

In this paper, we demonstrated that the combination of neutral loss scan of MS–MS with SIM and product scan of LC–MS–MS is feasible for fast screening and determination of certain classes of compounds in biological samples. This method can also be used for the analysis of other *O*-glycosides (for example, using neutral loss scan for the loss of m/z 162 to seek out glucosides), but not C-glycosides, whose glucosidic bond are unlikely to break under negative ion mode.

Acknowledgements

This work was financially supported by Chinese National Key foundation of Science (Grant No. 1999054404).

Table 2
The regression equations[a], linearity, detection limits[b] and retention times of compounds I, II, and III for the quantitative LC–MS–MS analysis of the extract of *Erigeron breviscapus*

Compound	Regression equation	Linearity	Detection limit (pg/ml)	Retention time (min)
I	$A=4.32\times10^5C+276$	0.992	30	15.1
II	$A=6.85\times10^6C-57.5$	0.997	15	13.4
III	$A=1.35\times10^6C-143$	0.995	110	9.7

[a] Peak-area, A, versus concentration, C, in μg/ml.
[b] The detection limit is the concentration corresponding to the signal projected to give a signal-to-noise ratio (S/N) of 3:1.

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