Talanta 144 (2015) 341–348

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Stable isotope labeling assisted liquid chromatography–electrospray tandem mass spectrometry for quantitative analysis of endogenous gibberellins

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ARTICLE INFO

Article history:
Received 4 April 2015
Received in revised form
17 June 2015
Accepted 20 June 2015
Available online 24 June 2015

Keywords:
Gibberellins
HPLC–ESI-MS/MS
Stable isotope labeling

ABSTRACT

In the current study, we developed a stable isotope labeling strategy for the absolute quantification of gibberellins (GAs) by high performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–ESI-MS/MS). *N,N*-dimethyl ethylenediamine (DMED) and its deuterated counterpart d^4-DMED were used to derivatize GAs extracted from plant tissue samples and GA standards respectively. The both derivatives of GAs were mixed and then subjected to HPLC–ESI-MS/MS analysis. The absolute quantification of GAs in plant tissues could be achieved by calculating the peak area ratios of DMED labeled GAs/d^4-DMED labeled GAs. In the proposed strategy, the derivatization reaction of the labeling reagents with GAs could be completed rapidly (within 5 min) with high efficiency (> 99%) under mild conditions. The resulting derivatives could produce specific fragments in collision induced dissociation (CID), leading to high selectivity in multiple-reaction monitoring (MRM) mode, thus enhanced the reliability of the LC–MS/MS method. Furthermore, the limits of quantitation (LOQs) of GAs were considerably decreased (2–32 folds) due to incorporating easily ionized moieties into GAs, and the quantification of GAs in plant tissue could be achieved without isotopically labeled GA standards. Good linearity was obtained with correlation coefficients R^2 values of > 0.99. The limits of detection (LODs) and quantitation (LOQs) ranged from 0.02 to 0.74 pg and 0.07 to 2.45 pg, respectively. Eleven GAs could be successfully determined in spiked sample with 72–128% recoveries and the relative standard deviations (RSDs) were between 1.0% and 13.9%. Finally, the developed method was successfully applied for the detection of GAs in 50 mg (fresh weight) *Oryza sativa* leaves.

1. Introduction

Gibberellins (GAs) are a class of acid phytohormones that regulate many aspects of plant growth and development, mainly including stem elongation, germination, flowering and fruit development [1–3]. Elucidation of GAs functions and the molecular mechanism of how GAs control the developmental processes of plants are important for agriculture production. Since the physiological actions of GAs partly depend on its endogenous concentrations, it is important to build a method for accurate quantification of GAs in plant samples.

Liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) has been used for GAs analysis due to its high selectivity and sensitivity [4–10]. However, the ionization efficiency of GAs in negative ion mode is poor, leading to the low sensitivity of GAs by LC–ESI-MS/MS. Most of the endogenous GAs in plant tissue exists in an extremely low level. In this case, it is difficult to get a full profile of the endogenous GAs by LC–ESI-MS/MS in negative ion mode. Furthermore, the complicated matrix of plant extracts leads to severe ionization suppression of GAs, and the ionization suppression varies widely depending on the co-elution. For this reason, isotopically labeled internal standards are essential to correct matrix effects during ionization. However, isotopically labeled internal standards of GAs are often expensive or difficult to obtain. Besides, the commercial available isotopically labeled internal standards of GAs only contain two deuterium atoms in structure, thus the cross interference derived from isotope forms of the native GAs is hard to avoid.

Stable isotope labeling has been proved to be an effective strategy for relative quantification of various kinds of analytes including plant hormones in samples with complex matrix [11–16]. Instead of synthesizing the stable isotope labeled internal standards of the analytes of interest, an alternative strategy is to introduce stable isotopes into analytes in comparative samples by

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http://dx.doi.org/10.1016/j.talanta.2015.06.056
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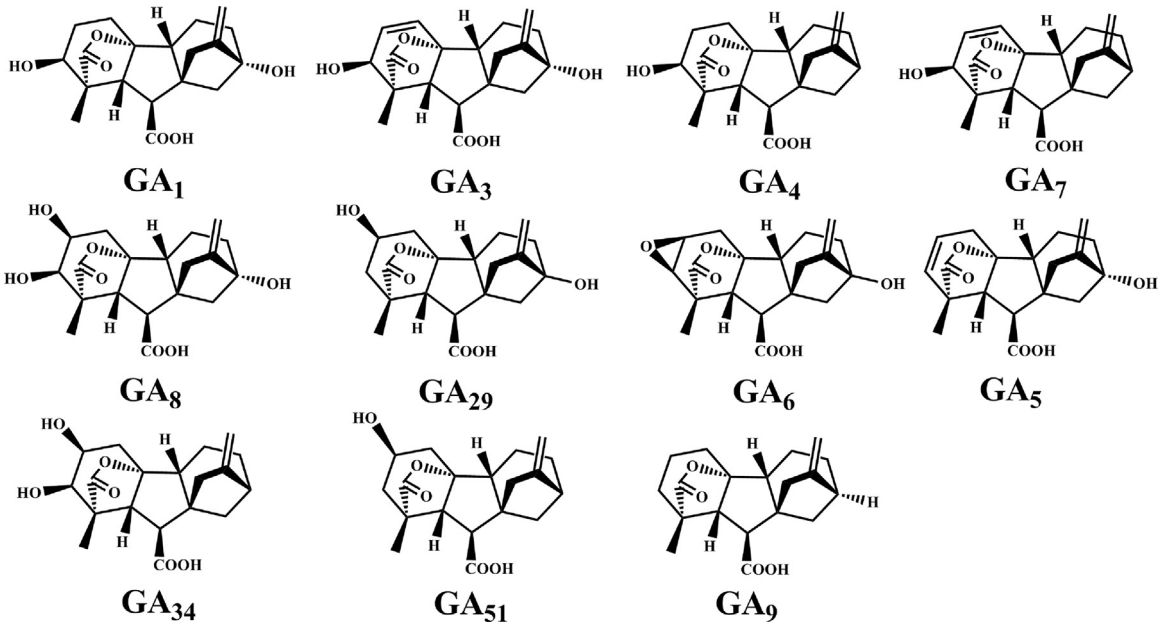

Fig. 1. Chemical structures of 11 GAs.

chemical derivatization. The two different labeled samples are mixed together and then subjected to LC–MS analysis. As the isotope labeled derivatives are expected to co-elute during the LC separation procedure, the matrix effects and run-to-run ionization differences are identical for them. Therefore, the peak intensity ratio of the different isotope labeled analyte pair provides relative quantification of analyte in two comparative samples. When one of the samples is a standard solution with a known concentration, absolute quantification can be achieved without the use of individual isotopically labeled internal standards. For detection of analytes of low concentrations, an ideal labeling reagent should contain ionizable functional groups in structure. Thus sensitivity can be improved by incorporating easily ionized moieties into analytes. Up to now, stable isotope labeling method has not been found in application of absolute quantitative analysis of plant hormones including GAs.

In this study, *N,N*-dimethyl ethylenediamine (DMED) and its isotope-labeled counterpart d^4-DMED were developed as a pair of isotope mass probes for absolute quantitative analysis of 11 GAs (Fig. 1) in complex plant samples by LC–MS/MS. The derivatization reaction could be completed rapidly with high efficiency under mild conditions, and the resulting GAs derivatives were found to produce specific fragments which impart high selectivity for MRM analysis. The developed stable isotope labeling assisted LC–MS/MS method exhibited high sensitivity and accuracy in the quantification of GAs without using individual isotopically labeled GAs. Finally, the developed method was successfully applied for determination of GAs in plant tissue samples, demonstrating its excellent application potential in plant hormone research.

2. Materials and methods

2.1. Chemicals and reagents

GA_S standards: GA_8, GA_{29}, GA_3, GA_1, GA_6, GA_5, GA_{34}, GA_{51}, GA_7, GA_4 and GA_9 were purchased from Olchemim Ltd. (Olomouc, Czech Republic). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Milli-Q water (Millipore, Bradford, USA) was used in all experiments. Formic acid (FA) and triethylamine (TEA), ethyl ether, 1,2-diaminoethane, ammonium formate, dichloromethane (DCM), methanol (MeOH) and acetic acid were bought from Sinopharm Chemical Reagent (Shanghai, China). Benzylorthochloroformiate, 10% palladium on activated charcoal, $NaBH_3CN$ and 2-chloro-1-methylpyridinium iodide (CMPI) were purchased from Aladdin Reagent Co. (Shanghai, China). *N,N*-dimethyl ethylenediamine (DMED, 98%) and deuterium formaldehyde (d^4-HCHO, 20% in water) were purchased from Sigma (St. Louis, MO, USA). C_{18} SPE cartridges (1 mL, 50 mg) were obtained from Weltech Co. (Wuhan, China).

The stock solutions of CMPI (100 μmol/mL), DMED (100 μmol/mL), d^4-DMED (100 μmol/mL) and TEA (100 μmol/mL) were prepared by dissolving appropriate amount of CMPI, DMED and TEA in ACN, respectively. For GA_8, GA_{29}, GA_3, GA_1, GA_6, GA_5, GA_{34}, GA_{51}, GA_7, GA_4 and GA_9, the stock solutions were prepared at the concentration of 10 μg/mL in ACN. All stock solutions were stored at −18 °C. The stock solutions were diluted with ACN to working solutions before analysis.

2.2. Synthesis of d^4-DMED

The pathway for synthesis of d^4-DMED is shown in Fig. 2.

2.2.1. Synthesis of (2-amino-ethyl)-carbamic acid benzyl ester

To a solution of 1,2-diaminoethane (30.0 g, 0.50 mmol) in DCM (500 mL) benzylorthochloroformiate (8.53 g, 50 mmol) in DCM

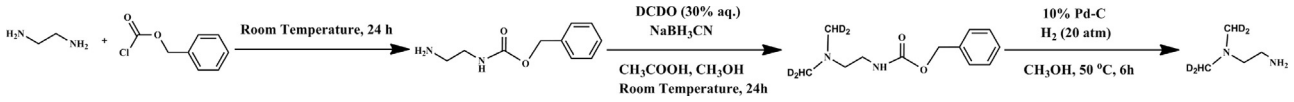

Fig. 2. Synthesis procedure of the d^4-DMED reagents.