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Short communication

Simultaneous quantification of individual intermediate steroids in silkworm ecdysone biosynthesis by liquid chromatography–tandem mass spectrometry with multiple reaction monitoring

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ABSTRACT

The concentration changes of endogenous ecdysteroids are closely related to the regulation of insect growth and development. Although they are frequently measured by immunoassays with anti-steroid antibodies, the separate estimations of the individual concentrations of ecdysone and other ecdysteroids with similar chemical structures are quite difficult to accomplish. In this study, an efficient method for the simultaneous, individual quantification of intermediate steroids in ecdysone biosynthesis was developed, using LC–MS/MS. By employing multiple reaction monitoring (MRM) in the MS detection, the selectivity and sensitivity of the method were greatly enhanced, allowing the estimation of trace amounts of steroids in biological samples from silkworm prothoracic glands and hemolymph.

1. Introduction

Ecdysteroids, such as ecdysone (E) and its metabolite, 20-hydroxyecdysone (20E), play important roles in insect growth, molting, and metamorphosis, as essential endogenous regulatory hormones [1]. The hormones are biologically synthesized, using cholesterol (C) as an exogenous material, during specific periods of insect growth [2,3]. In the prothoracic glands, which are the organs primarily responsible for ecdysone biosynthesis in insects, C is dehydrogenated to 7-dehydrocholesterol (7dC), and then 7dC is converted to 2,22,25-trideoxyecdysone (5β-ketodiol; KD) via several unknown reaction steps, which are currently referred to as a 'Black Box' [4,5] (for details of the ecdysone biosynthetic pathway, see Fig. S1 in the Supplementary materials). On the other hand, three cytochrome P450 monooxygenases that convert KD and its subsequent intermediates into E have been identified by molecular genetic analyses [3]. The enzymes sequentially hydroxylate KD at the 25-, 22-, and 2-positions, resulting in the production of 2,22-dideoxyecdysone (5β-ketotriol; KT), 2-deoxyecdysone (2dE), and E, respectively [6–8]. After its release from the prothoracic glands to the hemolymph, E is further converted to 20E by another monooxygenase in peripheral tissues [9].

To elucidate the regulatory mechanisms of insect metamorphosis by ecdysone biosynthesis, the concentration changes of the intermediate ecdysteroids in living insects must be monitored, but concise methods that can quantify the individual steroids simultaneously have not been developed. Among the ecdysteroids, the concentrations of E and 20E can be assessed, using radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) [10,11], but the steroids are not separately estimated with these methods, because the antibodies cannot discriminate between compounds with similar chemical structures. In addition, since specific antibodies against each intermediate ecdysteroid are not commercially available, such antibodies must be prepared in-house. To measure the amounts of individual steroids within the ecdysteroidogenic prothoracic glands, the similar ecdysteroids must be separated before quantification.

For the discriminable estimation of individual steroids, biological samples are often subjected to HPLC [12]. However, the elution conditions of the HPLC separation must be carefully determined,

Abbreviations: 2dE, 2-deoxyecdysone; 20E, 20-hydroxyecdysone; 7dC, 7-dehydrocholesterol; C, cholesterol; E, ecdysone; LLOQ, lower limit of quantification; KD, 5β-ketodiol (2,22,25-trideoxyecdysone); KT, 5β-ketotriol (2,22-dideoxyecdysone); MRM, multiple reaction monitoring; QC, quality control.

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because samples from the ecdysteroidogenic glands potentially contain steroids with similar structures but different hydrophobicities, ranging from the lipophilic C to the hydrophilic 20E. In addition, UV detection is not sensitive enough to quantify a picogram range of steroids in the samples, although most ecdysteroids have UV absorbance around 243 nm. In this study, to trace the steroid intermediates in the prothoracic glands and the hemolymph of growing silkworms, we devised an HPLC elution program suitable for the steroids, and employed mass spectrometry (MS) as a detector of the HPLC system. MS detection is much more sensitive than UV, and it was successfully utilized for the detection of ecdysteroids in previous reports [13–15].

2. Materials and methods

2.1. Materials

Among the standard steroids, C, 7dC, E, and 20E were obtained from Sigma–Aldrich (St. Louis, MO), and their purities were >99%, >99%, >90%, and >93%, respectively. KD and KT were chemically synthesized by Dr. Yoshinori Fujimoto, according to the previously published method [16], and 2dE was isolated and purified from silkworm ovary [17]. For these three steroids, the purities were >98%, >98%, and >97%, respectively, as estimated by HPLC analyses. All steroids were dissolved in methanol at a concentration of 1 mg/mL, and the solutions were diluted with methanol to obtain a series of standard solutions at different final concentrations, from 0.1 to 1000 ng/mL. All standard solutions were stored at −20 °C until use.

HPLC-grade acetonitrile was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

2.2. Conditions for the LC–MS/MS analyses

For the LC–MS/MS analyses, a Prominence gradient HPLC system (Shimadzu Corp., Kyoto, Japan) was used in combination with a triple quadrupole QTRAP® 5500 mass spectrometer (AB SCIEX, Framingham, MA), which was equipped with a standard atmospheric pressure chemical ionization (APCI) ion source. Chromatographic separation was performed with a PEGASIL ODS column (3 μm, 2.0 mm × 50 mm, Senshu Scientific Co., Ltd., Tokyo, Japan) at a flow rate of 0.4 mL/min, using a quite complex gradient elution protocol with acetonitrile/water (inset panel of Fig. 1). The MS detection of the steroids was conducted by multiple reaction monitoring (MRM) in the positive ion mode, for the simultaneous detection of the seven ecdysteroids: C, 7dC, KD, KT, 2dE, E, and 20E.

To verify the quantification method, recovery experiments were performed as described below, according to the Guidance for Industry (Bioanalytical Method Validation) published by the FDA [18]. As the matrix for preparing the calibration and quality control (QC) samples, a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2 mM EGTA (buffer A) was employed, because the actual samples from the silkworms may involve endogenous C and other steroids, and pure blank samples are not available from the insect tissues. QC samples at three different concentrations were prepared for each steroid by dissolving it in buffer A, and the steroid was extracted with two volumes of 1-butanol. On the other hand, the standard steroid solutions in methanol were used as references. The experiments were performed in triplicate, and the recovery yields of each steroid were calculated by comparing the LC–MS/MS MRM peak areas between the QC and reference samples. The experimental precision was expressed as the coefficient of validation (CV), and the accuracy was determined using the values obtained with the QC samples. For the intra-day validation, all of the samples in the triplicate experiments were examined on the same day, while they were analyzed on three different days for the inter-day validation.

For quantification of the steroids, a 10-μL portion of the standard mixture containing the seven steroids was injected, and calibration curves were generated with the steroids at appropriate concentrations: 10, 25, 50, 100, 250, and 500 ng/mL for C and 7dC; 0.25, 0.5, 1, 2.5, 5, 10, 25, and 50 ng/mL for KD, KT, and 2dE; and 0.1, 0.25, 0.5,1, 2.5, 5, 10, 25, and 50 ng/mL for E and 20E. Their peak areas were plotted and analyzed by linear least squares regression with weights of $1/x$, using the Analyst Software version 1.5 (AB SCIEX). According to the FDA Guidance [18], the concentration with acceptable precision (CV < 20%) and accuracy (deviation within ±20%) was employed as the lower limit of quantification (LLOQ) for the steroids, as long as they generated peaks with signal-to-noise (S/N) ratios greater than 5 in their MRM chromatograms.

2.3. Sample preparation from silkworms

Larvae of the silkworms, *Bombyx mori* (racial hybrid Kinshu × Showa), were reared with an artificial diet, Silkmate (Nihon Nosan Kogyo, Yokohama, Japan), at 25 °C under a 16 h-light/8 h-dark photoperiod.

To assess the steroids contained in the prothoracic glands, two pairs of the glands were dissected from fifth-instar larvae on different days, days-7 and 10, and they were sonicated at 4 °C for 10 min in 400 μL of buffer, containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2 mM EGTA. After the steroids were extracted with two volumes (800 μL) of 1-butanol, the organic fraction was collected and evaporated under reduced pressure to dryness [extraction procedure #4 in Fig. S2]. The residue was redissolved in 500 μL of methanol, and a 10-μL portion of the methanol solution was injected into the LC–MS/MS for quantification of the steroids.

On the other hand, to measure the steroid amounts in the silkworm hemolymph, the hemolymph was collected from the insects on all days, from the beginning of fourth-instar larvae to day-3 of pupa. According to the previous study [19], a 450 μL aliquot of methanol was added to 50 μL of the hemolymph, and the supernatant after centrifugation was subjected to the steroid quantification by LC–MS/MS.

3. Results and discussion

3.1. Mass spectrometry

To enhance the selectivity and sensitivity of the MS detection, multiple reaction monitoring (MRM) [20] was employed. To determine the best MRM parameters for the seven standard steroids, C, 7dC, KD, KT, 2dE, E, and 20E, which are all known intermediates in the ecdysone biosynthetic pathway (Fig. S1), their MS and MS/MS spectra were measured by the infusion of each steroid, one by one. For C and 7dC, the dehydrated molecular ions ($[M+H-H_2O]^+$), which are most abundant in their APCI-MS spectra, were selected as the precursor ions (Q1), while the molecular ions ($[M+H]^+$) themselves were selected for the other steroids. After fragmentation with the collision gas (N_2), most of the major fragments of the steroids were employed as the product ions (Q3) for their selective detection. The optimized parameters for the MRM detection are listed in Table 1.

3.2. Chromatography

The steroids were separated by reversed phase HPLC using an ODS column, with gradient elution by acetonitrile/water at a flow rate of 0.4 mL/min. For the best separation of the LC peaks of the seven ecdysteroids in a short period, the complex elution scheme was employed for the LC–MS/MS analyses (inset panel of Fig. 1).

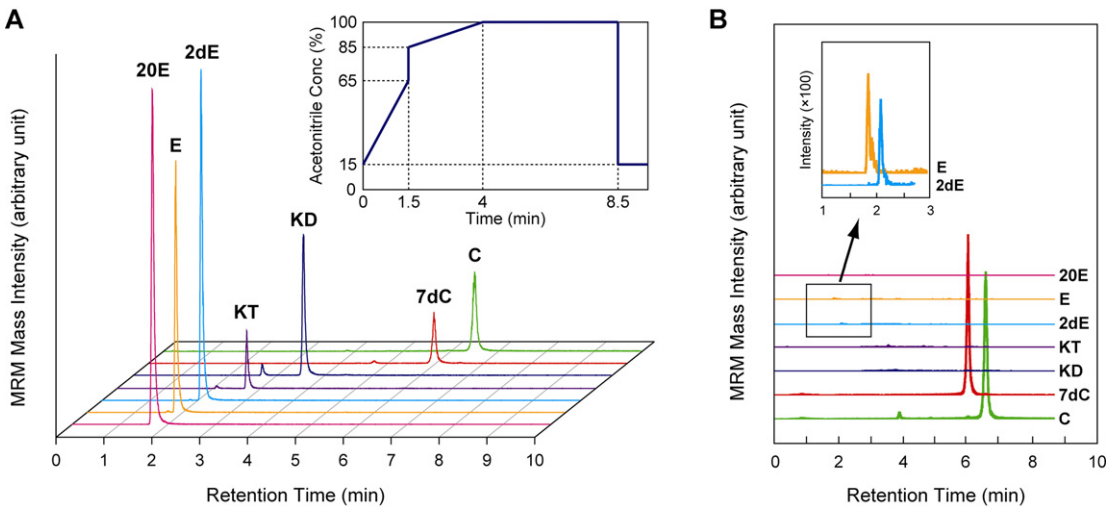

Fig. 1. Chromatograms of seven standard intermediate steroids in insect ecdysone biosynthesis, observed by LC–MS/MS with the MRM strategy. (A) Stacked chromatograms of the standard solution, containing 1.0 ng each of C (green), 7dC (red), KD (deep blue), KT (purple), 2dE (cyan), E (orange), and 20E (magenta), detected with steroid-selective MRM channels. The steroids were separated by reversed phase LC with a quite complex gradient elution with acetonitrile/water, as shown in the inset panel. (B) Typical chromatograms of a sample steroid extract from the prothoracic glands of a silkworm on day-10 of the fifth-instar (V10). To show the small peaks of E and 20E clearly, the corresponding parts of the same chromatograms are expanded 100-fold along the mass intensity axis in the inset figure.

With this scheme, one measurement cycle for the steroid quantification could be accomplished in less than 10 min. Fig. 1 shows typical chromatograms of the standard steroids and an actual sample from silkworm prothoracic glands, which were measured by MS detection with steroid-specific MRM channels. All of the steroids were well separated and almost specifically detected by the MRM protocol.

3.3. Method validation

Due to the unavailability of a pure blank matrix from the insect tissues, the matrix effects in the MS detection were evaluated in combination with the steroid recovery from the buffer solution, which was used for the extraction procedure of the steroids from silkworm prothoracic glands. The results of the recovery experiments ($n = 3$) are listed in Table S1 in the Supplementary materials. Although cholesterol (C), the most hydrophobic steroid among the analytes, showed quite low recovery yields under our experimental conditions, the others showed acceptable yields, which were more than 80%. The variation in the precision (CV values) for all of the steroids, including C, was less than 14% at every concentration examined, and the variation in the accuracy was at most ±13%. These results indicated that our LC–MS/MS method meets the requirements described in the FDA Guidance [18] for both the precision (CV < 20%) and accuracy (deviation within ±20%). The matrix effects may be practically negligible for these sample steroids.

Therefore, to prepare the calibration curves for each steroid, the standard solutions with appropriate amounts of the steroids were directly subjected to the LC–MS/MS measurements. As shown in Table 2, the calibration curves exhibited good linearity within the measured ranges of the standard steroids. The LLOQ values for the steroids, which were estimated based on the conditions described in Section 2, are also listed in Table 2. The obtained LLOQ values clearly indicated that the method can be applied to the insect ecdysteroid studies with sensitivity comparable to those of the conventional RIA and ELISA for E and 20E.

3.4. Quantification of the ecdysteroids in silkworm prothoracic glands

As the first application of the LC–MS/MS method to biological samples, the steroids contained in silkworm prothoracic glands were quantified, after optimization of the steroid extraction conditions (for the assessment of the extraction conditions, see Fig. S2 in the Supplementary materials). The glands were dissected from silkworms on days-7 and 10 of the fifth-instar larvae (abbreviated as V7 and V10, respectively, hereafter). The comparison of the ecdysteroid contents between the samples from different growth periods provided good verification of the ability of our quantification method by LC–MS/MS, since V7 and V10 are less and more activated periods of ecdysteroid biosyntheses in the silkworms, respectively. Among the ecdysteroids observed here, C and 7dC were most abundantly detected in the prothoracic glands (Fig. 2A). Since these two steroids are the primary precursors of ecdysteroidogenesis, they were constantly stored in higher amounts throughout the larval periods, including both V7 and V10, as previously reported [21,22]. In contrast, the amounts of 2dE and E dramatically increased at V10, which is the activated period of ecdysone biosynthesis, although they were still detected

Table 1
Optimized MRM parameters and LC retention times of the standard ecdysteroids.

Steroid	*m/z* value of precursor ion [Q1]	*m/z* value of product ion [Q3]	Declustering potential (eV)	Entrance potential (eV)	Collision energy (eV)	Collision cell exit potential (eV)	Retention time in LC (min)
Cholesterol (C)	369	147	70.9	13.3	30.9	13.9	6.5
7-Dehydrocholesterol (7dC)	367	145	71.0	10.0	21.0	8.0	6.0
5β-Ketodiol (KD)	417	381	96.0	10.0	23.0	32.0	3.6
5β-Ketotriol (KT)	433	397	90.0	10.0	25.1	8.4	2.7
2-Deoxyecdysone (2dE)	449	413	61.0	10.0	17.0	28.0	2.1
Ecdysone (E)	465	429	96.0	10.0	17.0	42.0	1.9
20-Hydroxyecdysone (20E)	481	445	91.0	10.0	23.0	10.0	1.7

Table 2
Linearity of calibration curves and LLOQ values for the ecdysteroids.

Steroid	Calibration concentration range (ng/mL)	Correlation coefficient	LLOQ (ng/mL)
Cholesterol (C)	10–500	0.997	10
7-Dehydrocholesterol (7dC)	10–500	0.993	10
5β-Ketodiol (KD)	0.25–50	0.999	0.25
5β-Ketotriol (KT)	0.25–50	0.998	0.25
2-Deoxyecdysone (2dE)	0.25–50	0.999	0.25
Ecdysone (E)	0.1–50	1.000	0.1
20-Hydroxyecdysone (20E)	0.1–50	0.999	0.1

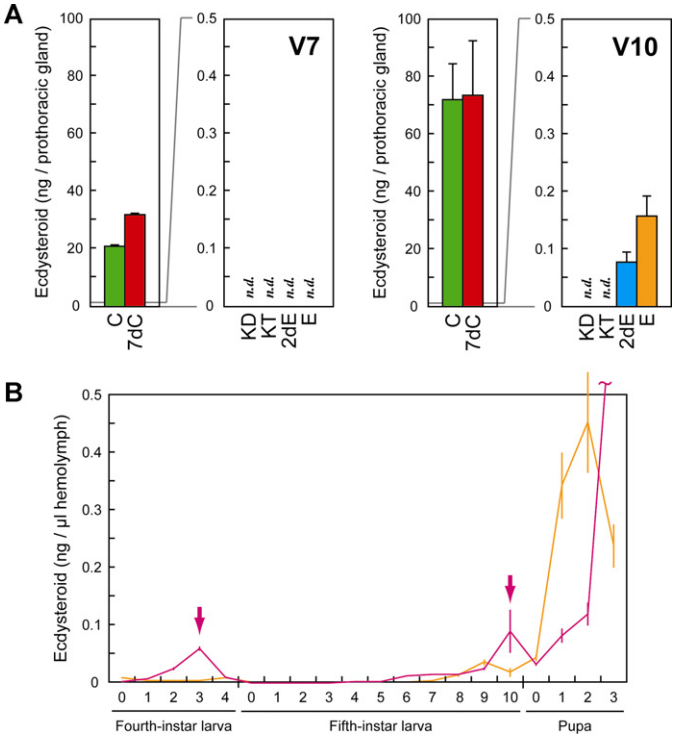

Fig. 2. (A) Quantification of the ecdysteroids in silkworm prothoracic glands on day-7 (V7) and day-10 (V10) of the fifth-instar larvae. Data of C, 7dC, 2dE, and E, which are shown as green, red, cyan, and orange bars, respectively, represent the mean ± standard error values per one prothoracic gland in three independent experiments. The abbreviation *n.d.* means 'not detected'. (B) Changes of the E (orange line) and 20E (magenta line) concentrations in silkworm hemolymph during periods from the fourth-instar larva to the pupa. Concentration data represent the mean ± standard error values of five silkworms. Two small but readily apparent peaks of the 20E concentration observed late in the fourth and fifth instars are shown by arrows.

at much lower levels in the glands than C and 7dC. No other intermediates, such as KD and KT, were detected in the prothoracic glands by our LC–MS/MS method. As the conversion reactions in the ecdysteroidogenesis are very fast, no detectable amounts of the intermediates may have remained in the glands.

3.5. Quantification of the ecdysteroids in silkworm hemolymph

On the other hand, E synthesized in the prothoracic glands is usually secreted from the glands to the hemolymph, and it is further converted into 20E by another monooxygenase in peripheral tissues [9] (Fig. S1). The metabolite, 20E, is more active for insect metamorphosis than E, but it has been difficult to measure separately from E. In this study, the changes of the E and 20E concentrations in hemolymph were separately measured by the LC–MS/MS method, as the silkworms grew from fourth-instar larvae to pupae (Fig. 2B). In the larval periods, small but readily apparent peaks of the 20E concentration were observed on day-3 of the fourth instar (IV3) and day-10 of the fifth instar (V10), as indicated by the arrows in Fig. 2B, while the E concentration was hardly affected on those days. Although previous studies reported similar changes in the ecdysteroid concentrations without discrimination between E and 20E [19,23], our LC–MS/MS method clearly revealed the differences in their peaks before the molting and enclosing of the silkworms. In the pupal period, the concentrations of E and 20E also showed different changes in hemolymph, and they were detected at much higher levels than those in the larvae. For instance, E showed a concentration peak on day-2, while 20E increased after the E peak.

4. Conclusion

Our method employing the MRM strategy successfully achieved the simultaneous quantification of all seven known intermediate ecdysteroids in biological samples from living silkworms. Although the concentrations of individual steroids can be estimated by immunoassays of HPLC-separated fractions [15], such procedures are much more laborious and time-consuming than our new method. The methodology provides a very concise and useful tool for studying insect metamorphosis, based on hormone biosynthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.12.014.

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