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Application of liquid chromatography–electrospray ionization mass spectrometry for study of steroid-converting enzymes

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Abstract

A high-performance liquid chromatography–atmospheric pressure ionization–electrospray ionization mass spectrometry (HPLC–API–ESI–MS) method was developed for the analysis of steroids in a study of steroid-converting enzymes. Separations ware done on a Zorbax Eclipse XDB-C18 column (eluted with a linear methanol–water–acetic acid gradient) and identification of the steroids involved was done by API–ESI–MS using positive ion mode and extracted ion analysis. The applicability of the present method for studying steroid metabolism was proven in assaying two steroid-converting enzymes (20β- hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase) in various biological samples (rat and chicken intestine, chicken oviduct).

Keywords: Steroids; Enzymes, steroid-converting

1. Introduction

Steroids represent compounds of significant biological and physiological influence on the animal body. They take part in the physiological processes in the organisms not only as endogenous compounds, but also as exogenous substances, such as drugs (typically antirheumatics) inclusive drugs of abuse. The role and function of steroids in the organism is complex indeed.

The biological role of steroids depends not only on their concentration in the plasma and on their interaction with relevant receptors, but also on the local metabolism of steroids in the target tissue. This type of metabolic pathway leads both to an increase or decrease of biologically active steroids depending on the balance between degradation and production of particular hormone. Glucocorticoids (cortisol in humans, corticosterone in rats and mice) and mineralocorticoids (aldosterone, deoxycorticosterone) possess similar affinity to the corticoid receptors in vitro, however in vivo the specific interactions differ. For this reason some mechanisms servicing specific interaction have to exist. It seems that this function is provided by 11β-hydroxysteroid dehydrogenase (E.C. 1.1.1.146). This enzyme changes local concentration of glucocorticoids by converting them to the 11-oxo-derivates that have lower affinity to the receptors. Another mechanism of corticoid receptor protection towards incorrect binding could be a conversion of the 20-oxo group to the 20-hydroxy group induced by 20-hydroxysteroid dehydrogenase.

In studying steroids, their influence on the organism, metabolism and further conversion, it is necessary to have a powerful technique for their analysis. The most comprehensive review on this subject is a book by Makin et al. [1]. The most frequently exploited methods for steroid analysis are gas chromatography (GC) and high-performance liquid chromatography (HPLC), as reviewed recently by Shimada et al. [2]. While GC–MS has been generally applied for steroid analysis and their biological relevance, HPLC with UV absorbance detection was applied to selected mixtures only [1]. The reason of this stems from the UV absorbance properties of steroids—only those which contain conjugated dienes and trienes, unsaturated ketones or aromatic chromophores have a good UV absorbing properties (typically corticosterone, cortisol or aldosterone). Naturally, it is possible to derivatize (both in the pre- or post-column) these steroids exploiting non-specific

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UV absorption and improve their UV or fluorescence detectability [1,3]; hydrazones (for oxo compounds) or post-column detection with sulphuric acid [1,3,4] represent typical examples. Capillary electrophoresis is another method for the separation of steroids. Various modes, as micellar electrokinetic chromatography or microemulsion electrokinetic chromatography [5–8] can be used for this purpose.

Capillary electrochromatography represents another possibility for steroid analysis. Two operational modes can be used, namely separation in columns (capillaries) packed with sol–gel bonded ODS/SCX [9] or on functionalized macroporous polyacrylamide gels [10,11] (for review see [12,13]). Separation on Zorbax ODS packed capillaries is also possible [14].

Recently the HPLC–MS methodology has been the relatively frequently used approach for steroid analysis. It can be exploited both for the analysis of free steroids and conjugates (sulphates or glucuronides) [15–22]. Regarding corticosterone, HPLC–thermospray mass spectrometry [15–17], HPLC–atmospheric pressure chemical ionization mass spectrometry [18] or HPLC–electrospray ionization (ESI) mass spectrometry [23] methods can be found in the literature. As an example of the GC–MS analysis of corticosterone the paper of Shu et al. [24] can be mentioned.

Numerous other reports deal with steroid analysis exists, Shibasaki et al. [15] used HPLC–thermospray mass spectrometry method for the quantitation of four corticosteroids in plasma, Fiori et al. [18] applied HPLC–atmospheric pressure chemical ionization mass spectrometry to corticosteroids used as illegal feed additives. A number of additional papers exists, however, they are dealing with the analysis of a single analyte (frequently a synthetic species) or steroids which were outside of interest of this communication [19–22,25].

In this paper we developed and applied a method for HPLC–API–ESI–MS assay of activity of steroid-converting enzymes. This work is a continuation of our investigation of these enzymes (mainly 11β-hydroxysteroid dehydrogenase) in animal intestines (rat, guinea pig or hen) [6,26–31]. Steroid standards were selected according to meet the requirements for steroid metabolites in these enzymatic process.

2. Experimental

2.1. Chemicals and reagents

Pregn-4-ene-21-ol-3,11,20-trione (11-dehydrocorticosterone), pregn-4-ene-11β,21-diol-3,20-dione (corticosterone) were purchased from Sigma (St. Louis, MO, USA); pregn-4-ene-20β,21-diol-3,11-dione (20-dihydro-11- dehydrocorticosterone), pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone), pregn-4-ene-21-ol-3,20-dione (deoxycorticosterone), pregn-4-ene-3,20-dione (progesterone), pregn-4-ene-20α-ol-3-one (20-dihydroprogesterone), 5α-androstan-3β,17β-diol, 5α-androstan-17β-ol-3-one (androstanolone), 5α-androstan-3α,17β-diol and pregn-5-ene-3β-ol-20-one (pregnenolone) were from Steraloids (Newport, RI, USA).

Methanol was HPLC gradient grade from Merck (Darmstadt, Germany), all other chemicals were purchased from Lachema (Brno, Czech Republic) in p.a. quality; Milli-Q (Millipore, Bedford, MA, USA) water was used throughout this study.

2.2. Instrumentation

The apparatus used was a HP 1100 LC/MSD system (Hewlett-Packard, Palo Alto, CA, USA) system consisting of a degasser, a binary pump, a autosampler, a thermostatted column compartment, a diode array detector and a mass detector LC/MSD.

2.3. Chromatography

Chromatographic separation was carried out on the Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm i.d., 5 μm, Rockland Technologies (Hewlett-Packard)). A 1 μl sample (dissolved in 1% acetic acid in methanol) was injected. Elution was achieved by a linear gradient between mobile phase A (methanol–water–acetic acid 40:60:1, v/v/v) and B (methanol). Gradient started from 10 to 100% B at 50 min with flow-rate 0.25 ml/min, then the column was eluted with 100% B for 5 min at elution flow 0.4 ml/min. Equilibration before the next run was achieved by 10 min washing with buffer A. Column temperature was held at 25 °C.

2.4. API–ESI mass spectrometry

Atmospheric pressure ionization–electrospray ionization (API–ESI) positive mode mass spectrometry was used. Operating conditions were optimized by FIA and were determined as follows: drying gas (N_2), 10 l/min; drying gas temperature, 350 °C; nebulizator pressure, 20 psi (138 kPa); capillary voltage, 4500 V; ions were observed at mass range *m*/*z* 200–500; fragmentor was set at 80 V. Reconstructed ion chromatogram for selected ion was set considering the whole mass range of the considered ion, e.g. for *m*/*z* 315, the mass range *m*/*z* is 315–316.

2.5. Animals

Experiments were performed on intestinal segments of male Wistar rats (age 80 days) and intestine or oviduct of Brown Leghorn chickens (age 30 days). The rats received a standard diet STS-1; chickens were fed by standard K-diet and all animals had free access to distilled water. These conditions were maintained since birth.

2.6. Assay of corticosterone metabolites in tissue fragments

Incubation of the tissue with the respective steroid was made according Vylitová et al. [6]. Briefly, studied steroid (1.45 μM) was incubated with the tissue slices (300 mg) in sealed vessels containing 10 ml of oxygenated incubation solution (in mM): NaCl, 119.0; $CaCl_2$, 1.2; $MgCl_2$, 1.2; $NaHCO_3$, 21.0; K_2HPO_4, 2.4; KH_2PO_4, 0.6; glucose, 10.0; glutamine, 2.5; β-hydroxybutyrate, 0.5; and manitol, 10.0; previously gassed for 10 min with 95% O_2/5% CO_2, pH 7.4 for 80 min at 37 °C. At the end of the incubation internal standard (deoxycorticosterone) was added (1.45 μM) and the vessels placed on ice. The incubation medium was centrifuged at 3000 × *g* for 10 min. The pellet was stored for calculation of dry weight and the supernatant was passed through the Sep-Pak C18 cartridge (Waters, Milford, MA, USA). Prior to use the cartridge was conditioned by passing through 5 ml of methanol and 5 ml of distilled water.

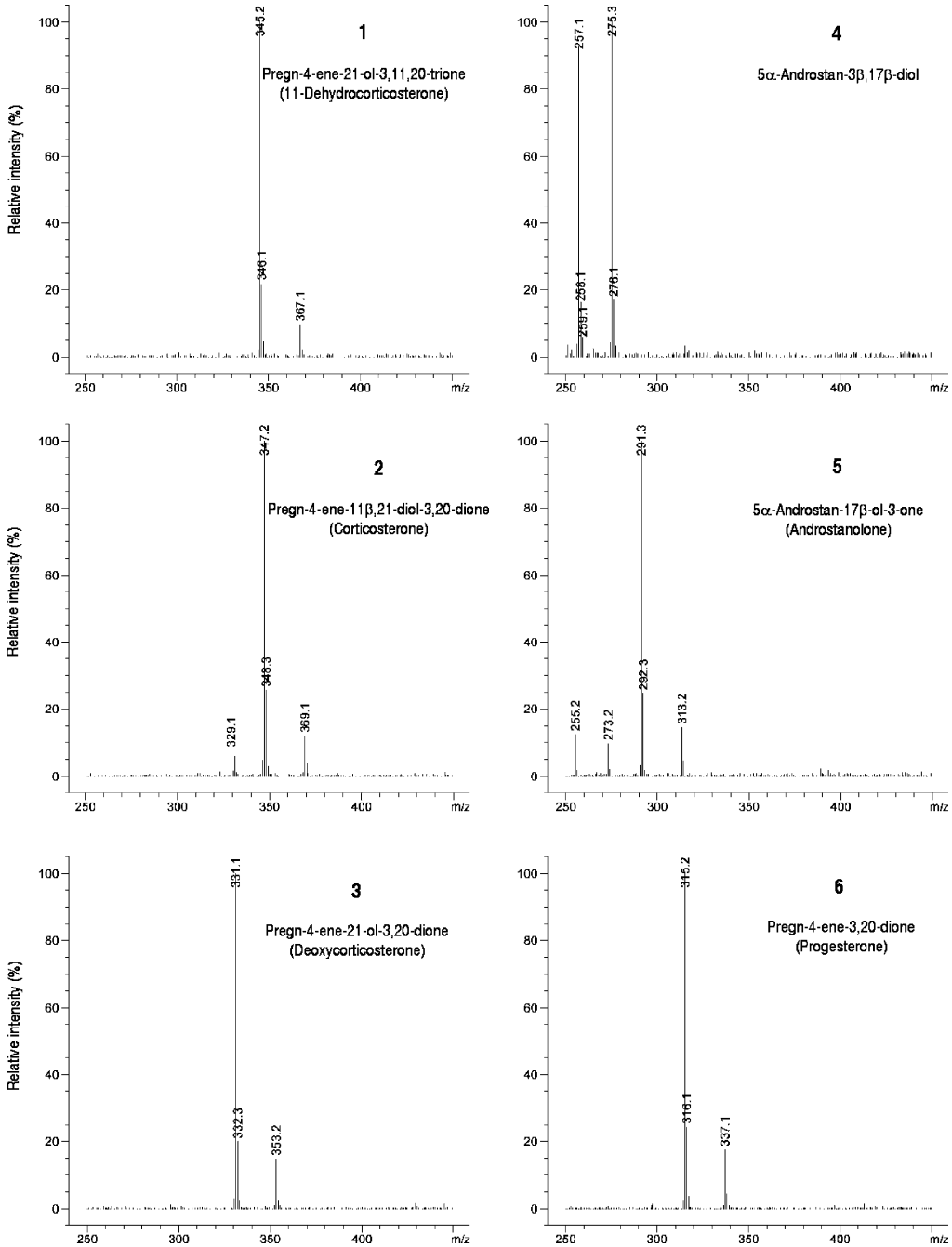

Fig. 1. API–ESI mass spectra of steroid metabolite standards.

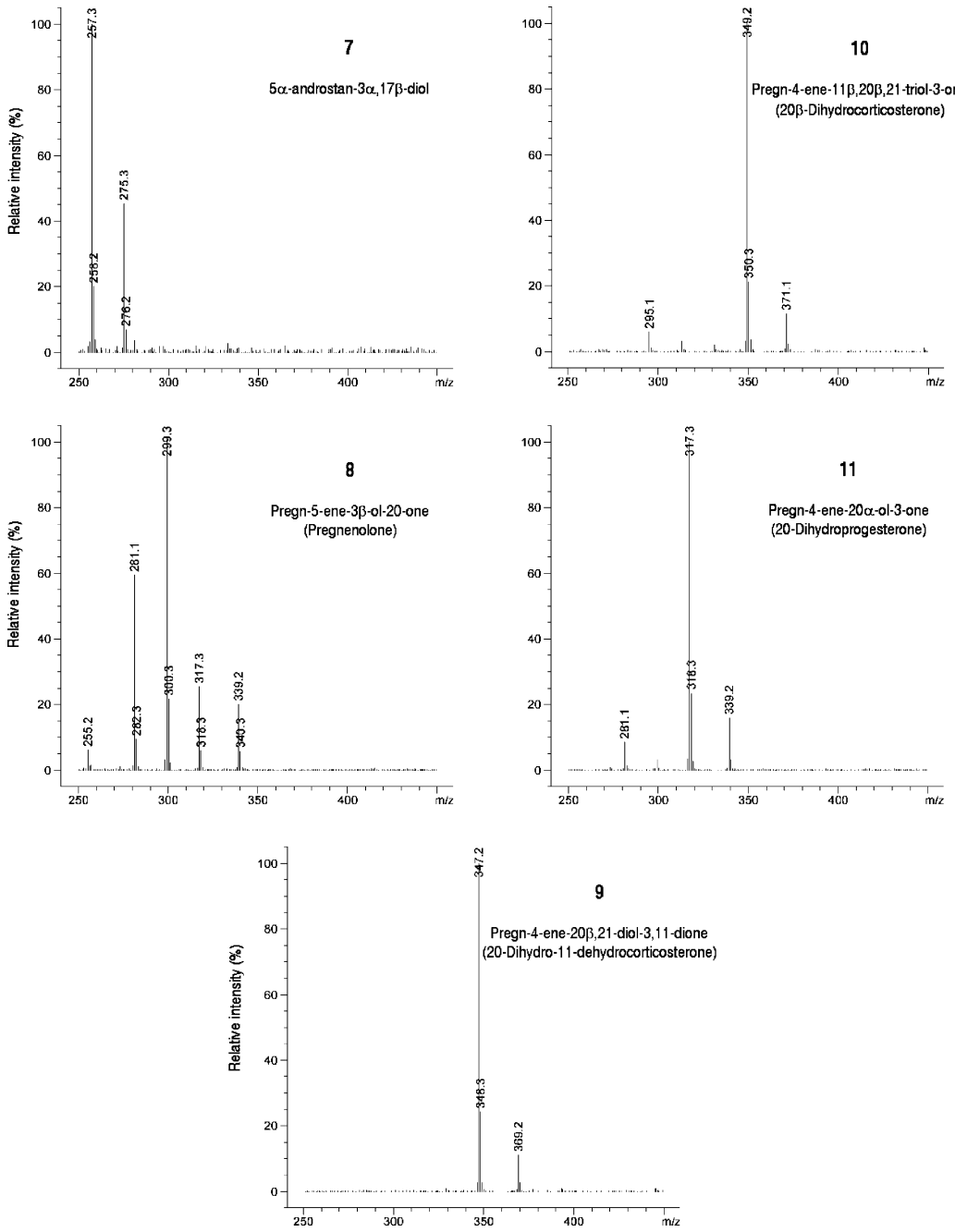

Fig. 1. (*Continued*).

After introduction of the sample, undesired compounds were removed by rinsing with water (4 ml) and steroids were eluted by 2 ml methanol. This eluate was evaporated to dryness under nitrogen and the residue was reconstituted in 20 μl methanol with 1% acetic acid for analysis.

2.7. Statistical analysis

Data in the application part are presented as the mean ± S.E.M. Activity of the enzyme was expressed as nanograms of product per milligrams of dry weight per hour (ng/(mg DW h)).

3. Results and discussion

3.1. Developing of method and separation of standards

In the present work we attempted to separate a mixture of steroids applied for analysis of metabolic products of glucocorticoids (namely corticosterone). UV detection was ineffectual in these separation from two reasons: (1) not all compounds of interest have acceptable UV properties (induced by, e.g. conjugated dienes); (2) in the complex mixture of biological samples it is often very difficult to make decision about the presence of steroid(s) in the mixture

Table 1
Peak identification, extracted ions used for quantitation and retention times

Peak no.	Compound	Extracted ion (*m/z*)	Retention time (min)
1	Pregn-4-ene-21-ol-3,11,20-trione (11-dehydrocorticosterone)	345	7.3
9	Pregn-4-ene-20β,21-diol-3,11-dione (20-dihydro-11-dehydrocorticosterone)	347	9.1
2	Pregn-4-ene-11β,21-diol-3,20-dione (corticosterone)	347	10.6
10	Pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone)	349	12.2
3	Pregn-4-ene-21-ol-3,20-dione (deoxycorticosterone)	331	15.2
4	5α-Androstan-3β,17β-diol	275	18.8
5	5α-Androstan-17β-ol-3-one (androstanolone)	291	20.1
6	Pregn-4-ene-3,20-dione (progesterone)	315	22.7
7	5α-Androstan-3α,17β-diol	257	23.6
8	Pregn-5-ene-3β-ol-20-one (pregnenolone)	299	26.2
11	Pregn-4-ene-20α-ol-3-one (20-dihydroprogesterone)	317	26.7

according to the retention time only (and quantification could be difficult problem as well). In this case it appeared useful to compare mass spectrum of the analyzed compound with standards. Coupling of HPLC with MS and post-separation analysis by ion extraction method (and mass spectra) allows to determine and quantify steroids that are not well resolved [32].

Three compounds of mixture of selected steroids (5α-androstan-3β,17β-diol, 5α-androstan-17β-ol-3-one (androstanolone) and 5α-androstan-3α,17β-diol) are not detectable by UV detection. Mass spectra of individual standards are shown in Fig. 1. Selection of characteristic ions for extracted ion analysis allowed to identify and quantify all investigated metabolites (Fig. 2, Table 1). As shown in Fig. 2, good separation was obtained for compounds with the same molecular mass or characteristic ions, as α and β isomers of two compounds (nos. 4 and 7). These results offered the possibility to use the present method for both qualitative and quantitative analysis.

The single problem could arise in the case of separation (identification) and quantitation of compounds nos. 11 (pregn-4-ene-20α-ol-3-one; 20-dihydroprogesterone) and 8 (pregn-5-ene-3β-ol-20-one; pregnenolone). It is obvious from Fig. 2 that these steroids have a similar retention time. If we analyze the mixture (a biological sample) that contains one of these compounds, it is impossible to carry out identification on the basis of the retention time only. In this case it is useful to apply the method of extracted ions. When we look at the spectra of both compounds we can see some problem in identification. In the case of pregnenolone the characteristic ion 299 *m/z* that is not present in the 20-dihydroprogesterone mass spectra can be used. Ion 317 *m/z* that was used for determination of 20-dihydroprogesterone is on the contrary present also in the spectra of pregnenolone. In this case the method of extracted ions can be used with simultaneous comparison of retention times on the same chromatographic record. The principle of this method is demonstrated on Fig. 3. Identification (and determination) of pregnenolone was based on using the ion *m/z* 299. The *m/z* 317 ion was used for identification of 20-dihydroprogesterone keeping in mind that the response for pregnenolone is significantly lower (about 5% in comparison the *m/z* 299 ion). Simultaneously both these compounds differ in retention time. Therefore it is possible to use ion *m/z* 299 for determination of pregnenolone, then the compound in question is. If the content of pregnenolone is higher, it can be revealed by the ion *m/z* 317. At the longer retention time (approximately 0.5 min) it is possible to identify and quantify 20-dihydroprogesterone by using ion *m/z* 317. Of course, verification of results can be completed by comparison of mass spectra.

Calibration curves (based on peak areas of selected extracted ions) were linear in the region 2–60 ng (four points, three repeated measurements, 5, 10, 50 and 100 ng of standards injected). The correlation coefficients were larger than 0.991 with a precision (expressed as coefficient of variation, CV) within 10%. Accuracy for spiked samples (recovery) fell within the 87.5–95.5%. Some typical examples of calibration curves ($y = a + bx$, where y is peak area and x the amount of steroid) are, for deoxycorticosterone $y = 1 \times 10^3 + 65 \times 10^3 x$, $r^2 = 0.9969$, and for progesterone $y = 2 \times 10^3 + 79 \times 10^3$, $r^2 = 0.9963$.

Detection limits (LODs) (signal-to-noise ratio 3:1) of the signal of the extracted ions were around 1 ng and refer to spiked samples. These limits are compatible with data reported by Shibasaki et al. [15] (1–0.25 ng) and Fiori et al. [18] (5–2 ng) for other steroids. A better limit could be obtained by using selected ion monitoring (SIM) (approximately 100 times), however in this case it is impossible to obtain structural information about compounds separated. In a complex biological mixture it may be important to have this information for the two main reasons: (1) owing to complexity of biological samples we can be sure that the compound in the particular retention time is the compound of interest (in the same retention time one can have many other compounds that contain the same *m/z* ion); (2) in studying of biological systems any information about unpredicted newly arising compounds would be considerable value. Of course, when a new metabolite is observed (and

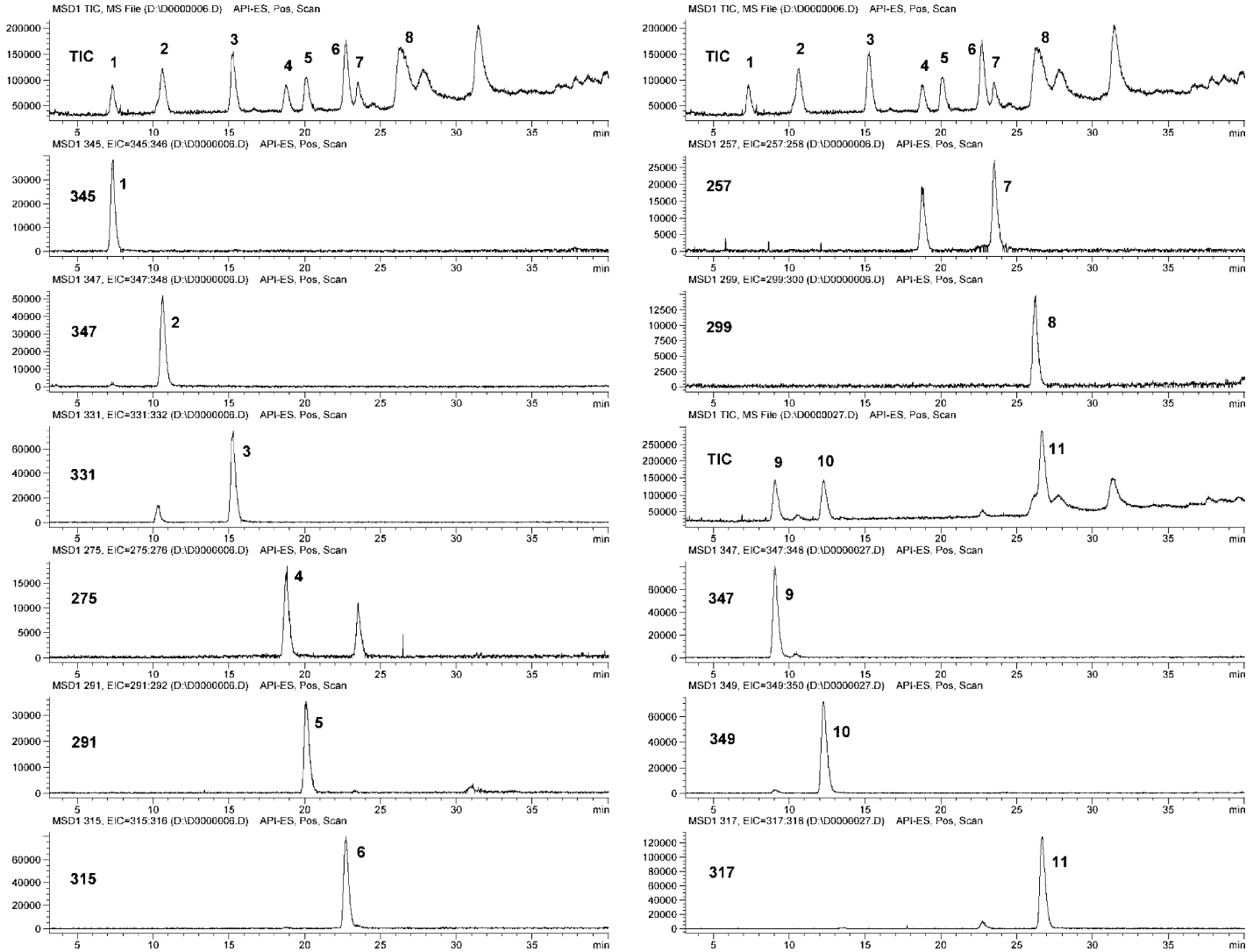

Fig. 2. Separation of a standard mixture of steroids, monitored by extracted ions. Total ion current (TIC) of 200–500 *m/z* for mixture of standards 1–8 (first line at the left column) and 9–11 (fourth line at the right column), specification of individual ions (*m/z*): 345, pregn-4-ene-21-ol-3,11,20-trione (11-dehydrocorticosterone) (1); 347, pregn-4-ene-11β,21-diol-3,20-dione (corticosterone) (2) and pregn-4-ene-20β,21-diol-3,11-dione (20-dihydro-11-dehydrocorticosterone) (9); 331, pregn-4-ene-21-ol-3,20-dione (deoxycorticosterone) (3); 275, 5α-androstan-3β,17β-diol (4); 291, 5α-androstan-17β-ol-3-one (androstanolone) (5); 315, pregn-4-ene-3,20-dione (progesterone) (6); 257, 5α-androstan-3α,17β-diol (7); 299, pregn-5-ene-3β-ol-20-one (pregnenolone) (8); 349, pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone) (10); 317, pregn-4-ene-20α-ol-3-one (20-dihydroprogesterone) (11).

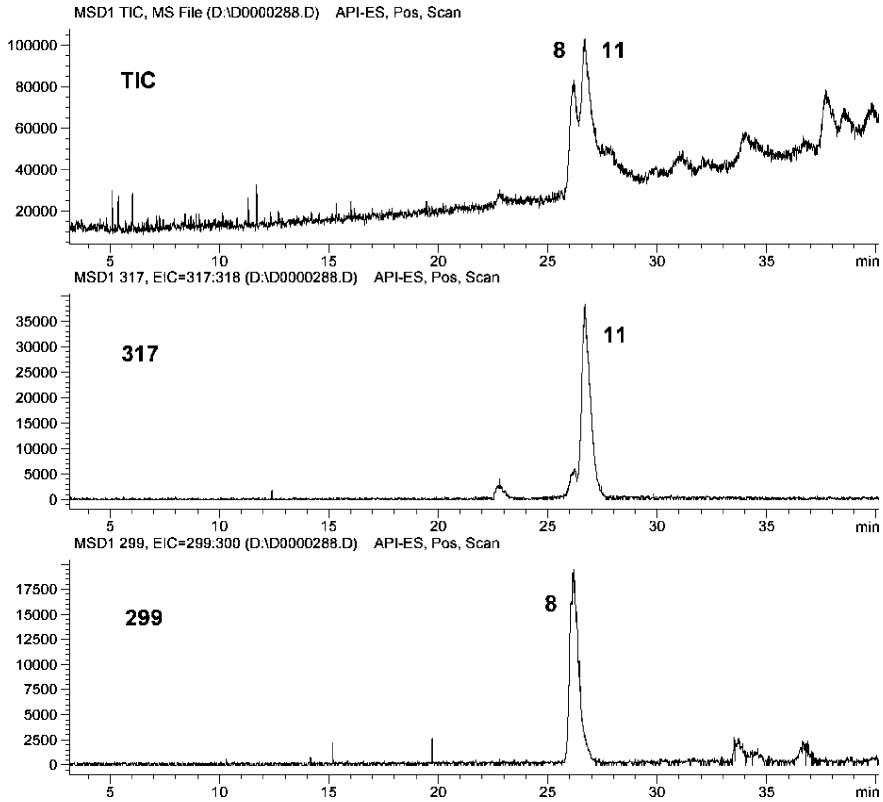

Fig. 3. Separation of pregn-5-ene-3β-ol-20-one (pregnenolone) (8) and pregn-4-ene-20α-ol-3-one (20-dihydroprogesterone) (11) and their analysis by total ion current (TIC) and using extraction ions (317 and 299). The amount of pregnenolone was twice higher than 20-dihydroprogesterone (100 and 50 ng, respectively).

detected) confirmation by mass fragments is needed in addition to the information about retention time. This was precisely the situation in our experiments concerning the activity of steroid dehydrogenases in the chicken oviduct (see Section 3.2).

ESI–MS spectra were characterised by the quasi-molecular ions $[M + H]^+$, sometimes accompanied by the $[M + H\text{-}18]^+$ and $[M + H\text{-}36]^+$ fragment ions, resulting from the loss of one and two water molecules respectively. Ions $[M + 23]^+$ are probably adduct ions corresponding to $[M + Na]^+$. This finding is in concurrence with results reported by Shibasaki et al. [15] with thermospray and Fiori et al. [18] with atmospheric pressure chemical ionization for various corticosteroids. Marwah et al. [23] also described the occurrence of $[M + 23]^+$ of $[M + Na]^+$ ion that they used for quantitation of corticosterone by SIM mode of API–ESI–MS. On the contrary, ion $[M + H\text{-}60]^+$ stemming from the loss of glycoaldehyde was not observed. Similar results were reported by Wiedmer et al. [7] for MECC–ESI–MS of four corticosteroids (cortisone, cortisol, corticosterone and 1-dehydroaldosterone).

3.2. Applicability

The applicability of the present method for the study of steroid metabolism was proven by assaying of steroid-converting enzymes. An example of chromatographic profiles from tissue slices is presented in Fig. 4. It is obvious that the applied method, extracted ions, enables to identify and quantify steroids in a relatively complex mixture of compounds present in the tissue.

Three experiments that used whole spectra of selected steroid standards regarding glucocorticoid metabolism are presented here as demonstrative examples.

The occurrence (and activity) of 11β-hydroxysteroid dehydrogenase in the rat intestine was studied with the results shown in Table 2. Corticosterone was used as substrate. The activity of this enzyme was determined in various segments of the intestine—the highest activity was present in colon and no activity was revealed in jejunum.

Table 2
Conversion of corticosterone to 11-dehydrocorticosterone (activity of 11β-hydroxysteroid dehydrogenase) in individual parts of tissue slices of rat intestine, concentration of corticosterone was 1.45 μM

Part of intestine	Activity of enzyme (ng/mg DW h)
Jejunum	0 (6)
Ileum	7.93 ± 1.38 (6)
Caecum	17.56 ± 2.41 (6)
Colon	21.00 ± 2.41 (6)

$N = 6$.

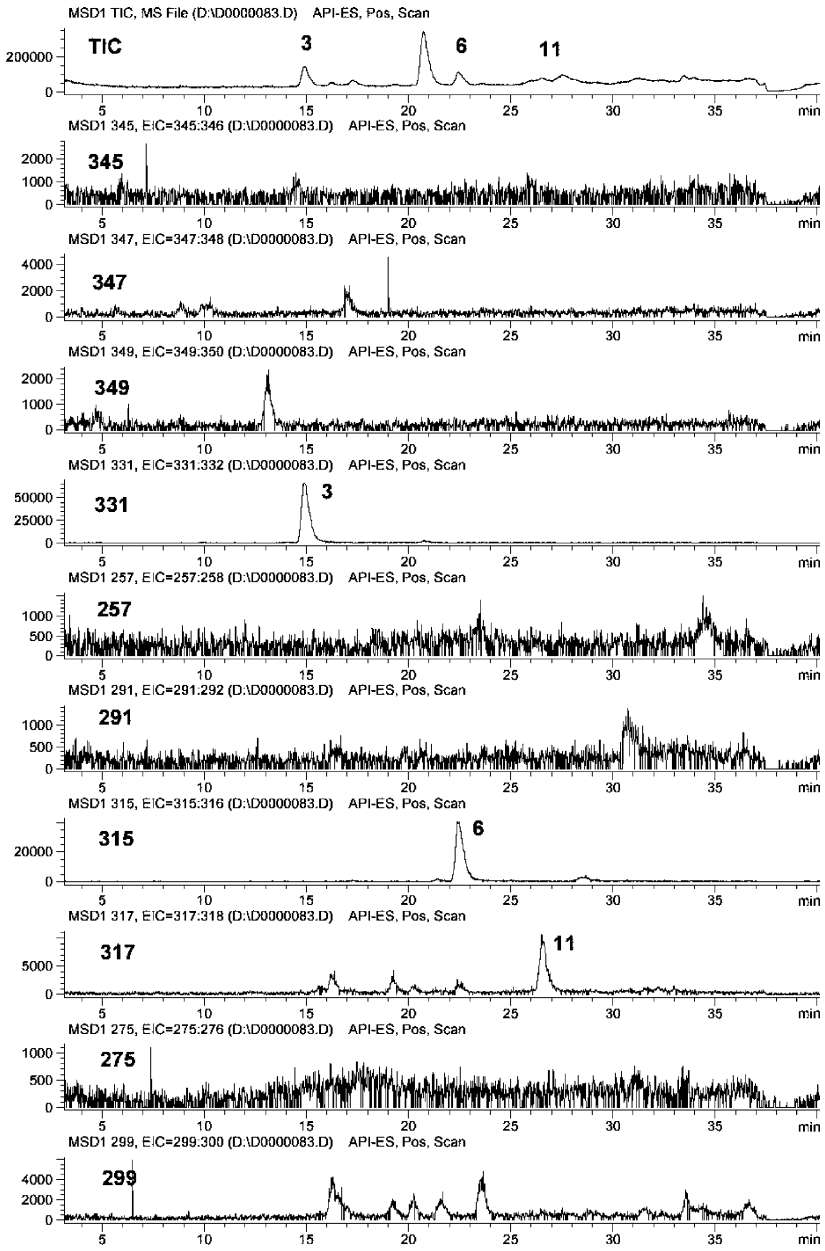

Fig. 4. Separation of steroid compounds in a tissue sample (study of conversion of progesterone to 20-dihydroprogesterone in individual parts of tissue slices of chicken intestine). Identification: 331, pregn-4-ene-21-ol-3,20-dione (deoxycorticosterone) (3); 315, pregn-4-ene-3,20-dione (progesterone) (6); 317, pregn-4-ene-20α-ol-3-one (20-dihydroprogesterone) (11).

In the avian intestine (on the contrary to the rat intestine), 20β-hydroxysteroid dehydrogenase is present [26]. Two substrates were used: corticosterone and progesterone. The presence (and activity) of 20β-hydroxysteroid dehydrogenase was proven by the determination of 20-dihydrocorticosterone or 20-dihydroprogesterone, respectively. It was demonstrated that the activity of the studied enzyme was significantly lower towards of progesterone (see Table 3).

The method developed was applied also for the study of steroid metabolism in the chicken oviduct after estrogens stimulation. On the contrary to the intestine no corticosterone was inactivated to 11-dehydrocorticosterone or 20-dihydrocorticosterone but 11-dehydrocorticosterone was reduced to corticosterone. This finding indicates in oviduct a low activity of 11β-hydroxysteroid dehydrogenase operating as a reductase. Androstanolone (5α-dihydrotestosterone), the principle male hormone, was converted to both 5α-androstan-3α,17β-diol and 5α-androstan-3β,17β-diol (Table 4). The observed reduction at C_3 indicates the presence of 3-hydroxysteroid dehydrogenase in chicken oviduct.

Table 3
Conversion of progesterone to 20-dihydroprogesterone and conversion of corticosterone to 20-dihydrocorticosterone (activity of 20-hydroxysteroid dehydrogenase) in individual parts of tissue slices of chicken intestine, concentrations of steroids were 1.45 μM

Part of intestine	Activity of enzyme (ng/mg DW h)
Progesterone	
Jejunum	0 (10)
Ileum	0.74 ± 0.31 (10)
Caecum	0.85 ± 0.36 (10)
Colon	4.02 ± 1.02 (9)
Corticosterone	
Jejunum	40.2 ± 4.8 (7)
Ileum	36.4 ± 3.8 (12)
Caecum	31.2 ± 3.0 (7)
Colon	29.5 ± 3.1(6)

Number of experiments in parenthesis.

Table 4
Activity of steroid dehydrogenases in the chicken oviduct after stimulation by estrogens using various substrates

Substrate	Arising product	Activity of enzyme (ng of product/mg DW h)
Androstanolone (5α-androstan-17β-ol-3-one)	5α-Androstan-3β,17β-diol	72.3 ± 13.8 (5)
	5α-Androstan-3α,17β-diol	195.4 ± 33.3 (5)
11-Dehydrocorticosterone	Corticosterone	10.7 ± 2.4 (5)

Number of experiments in parenthesis.

4. Conclusions

There was developed an HPLC–MS–API–ESI method for study of steroid-converting enzymes. This method allows to separate 10 steroids (and one standard) involved in steroid metabolism.

On the basis of this newly developed HPLC–MS method it is possible to conclude that (1) steroid metabolism exhibits significant interspecies differences in the same tissue, (2) there are tissue dependent differences in the metabolism of the same steroid, and (3) 20β-hydroxysteroid dehydrogenase in the avian intestine preferentially uses corticosterone (in comparison to progesterone) as substrate.

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References

[1] H.L.J. Makin, D.B. Gower, D.N. Kirk (Eds.), Steroid Analysis, Blackie, London, 1995.
[2] K. Shimada, K. Mitamura, T. Higashi, J. Chromatogr. A 935 (2001) 141.
[3] K. Blau, J. Halket (Eds.), Handbook of Derivatives for Chromatography, second ed., Wiley, Chichester, 1993.
[4] A. Sudo, J. Chromatogr. 528 (1990) 453.
[5] L. Vomastová, I. Mikšík, Z. Deyl, J. Chromatogr. B 681 (1996) 107.
[6] M. Vylitová, I. Mikšík, J. Pácha, Gen. Comp. Endocrinol. 109 (1998) 315.
[7] S.K. Wiedmer, M. Jussila, M.-L. Riekkola, Electrophoresis 19 (1998) 1711.
[8] C.-H. Wu, M.-C. Chen, A.-K. Su, P.-Y. Shu, S.-H. Chou, C.-H. Lin, J. Chromatogr. B 785 (2003) 317.
[9] Q. Tang, M.L. Lee, J. High Resolut. Chromatogr. 23 (2000) 73.
[10] A.H. Que, A. Palm, A.G. Baker, M.V. Novotny, J. Chromatogr. A 887 (2000) 379.
[11] C. Fujimoto, Y. Fujise, E. Matsuzawa, Anal. Chem. 68 (1996) 2753.
[12] I. Mikšík, Z. Deyl, in: F. Švec, T. Tennikova, Z. Deyl (Eds.), Monolithic Materials, Elsevier, Amsterdam, 2003, p. 632.
[13] Z. Deyl, I. Mikšík, in: Z. Deyl, F. Švec (Eds.), Capillary Electrochromatography, Elsevier, Amsterdam, 2001, p. 355.
[14] C. Huber, G. Choudhary, C. Horváth, Anal. Chem. 69 (1997) 4429.
[15] H. Shibasaki, T. Furuta, Y. Kasuya, J. Chromatogr. B 692 (1997) 7.
[16] S. Steffenrud, G. Maylin, J. Chromatogr. 577 (1992) 221.
[17] S.J. Park, Y.J. Kim, H.S. Pyo, J. Park, J. Anal. Toxicol. 14 (1990) 102.
[18] M. Fiori, E. Pierdominici, F. Longo, G. Brambilla, J. Chromatogr. A 807 (1998) 219.
[19] A. Polettini, G.M. Bouland, M. Montagna, J. Chromatogr. B 713 (1998) 339.
[20] S.J. Gaskell, K. Rollins, R.W. Smith, C.E. Parker, Biomed. Environ. Mass Spectrom. 14 (1987) 717.
[21] D. Barrón, J. Barbosa, J.A. Pascual, J. Segura, J. Mass Spectrom. 31 (1996) 309.
[22] K.A. Bean, J.D. Henion, J. Chromatogr. B 690 (1997) 65.
[23] A. Marwah, P. Marwah, H. Lardy, J. Chromatogr. B 757 (2001) 333.
[24] P.Y. Shu, S.H. Chou, C.H. Lin, J. Chromatogr. B 783 (2003) 93.
[25] Q.G. Wang, Z.P. Wu, Y.M. Wang, Anal. Lett. 34 (2001) 103.
[26] J. Pácha, I. Mikšík, Life Sci. 54 (1994) 745.
[27] J. Pácha, I. Mikšík, J. Endocrinol. 148 (1996) 561.
[28] I. Pohlová, I. Mikšík, J. Pácha, Mech. Ageing Dev. 98 (1997) 139.
[29] J. Pácha, I. Mikšík, V. Lisá, I. Pohlová, Life Sci. 61 (1997) 2391.
[30] I. Pohlová, I. Mikšík, J. Kuneš, J. Pácha, Am. J. Hypertens. 13 (2000) 927.
[31] J. Pácha, V. Lisá, I. Mikšík, Steroids 67 (2002) 119.
[32] I. Mikšík, M. Vylitová, J. Pácha, Z. Deyl, J. Chromatogr. B 726 (1999) 59.