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Separation and identification of corticosterone metabolites by liquid chromatography–electrospray ionization mass spectrometry

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Abstract

High-performance liquid chromatography coupled to atmospheric pressure ionization–electrospray ionization mass spectrometry (API–ESI–MS) was investigated for the analysis of corticosterone metabolites; their characterization was obtained by combining the separation on Zorbax Eclipse XDB C_{18} column (eluted with a methanol–water–acetic acid gradient) with identification using positive ion mode API–ESI–MS and selected ion analysis. The applicability of this method was verified by monitoring the activity of steroid converting enzymes (20β-hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase) in avian intestines.

Keywords: Corticosterone

1. Introduction

High-performance liquid chromatography (HPLC) coupled to atmospheric pressure ionization–electrospray ionization (API–ESI) mass spectrometry (MS) is gaining popularity among analytical techniques [1]. Complex mixtures of steroids have been traditionally analyzed mainly by gas chromatography (GC)–MS, while HPLC with UV absorbance detection was applied to selected mixtures only [2]; the reason is that UV absorbance can be used to reveal steroids which exhibit favorable UV absorbing properties based on the presence of conjugated dienes and trienes, unsaturated ketones or aromatic chromophores, typically corticosterone, aldosterone etc. (for details see Ref. [2]). A number of other steroids have to be derivatized (no matter whether in the pre- or post-column mode) to enable UV absorbance or fluorescence detection [2,3]; hydrazones (for oxo compounds) or post-column detection with sulfuric acid [2–4] represent typical examples. In addition to established analytical methods it is also possible to use capillary electrophoretic methods, namely micellar electrokinetic chromatography or microemulsion electrokinetic chromatography [5,6]. Recently the LC–MS analysis of a number of free steroids and their sulfate as well as glucuronide conjugates appeared in the literature [7–14]. The HPLC–thermospray (TSP)–MS [7–9] and HPLC–atmospheric pressure chemical ionization (APCI)–MS [10] methods for separation of corticosteroids have been described. Shibasaki et al. [7] developed a HPLC–TSP–MS method for the quantitation of four corticosteroids in plasma (cortisol, cortisone, prednisolone

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and prednisone) using stable isotope dilution. Fiori et al. [10] applied HPLC–APCI–MS to corticosteroids used as illegal feed additives (dexamethasone, betamethasone, flumethasone, triamcinolone, prednisone, prednisolone, methylprednisolone, fludrocortisone and beclomethasone). A number of additional papers were published dealing with the analysis of a single analyte (frequently a synthetic species) or steroids which were outside of interest of this communication [11–14]. Recently, combination of micellar electrokinetic capillary chromatography (MECC) with ESI–MS for the analysis of four corticosteroids (cortisone, cortisol, corticosterone and 1-dehydroaldosterone) was described by Wiedmer et al. [15].

In this paper we developed a method for HPLC–API–ESI–MS assay of corticosterone metabolites. Our previous studies of these glucocorticoids were directed to the investigation of enzymes (mainly 11β-hydroxysteroid dehydrogenase) in the animal intestine (rat, guinea pig and hen) [6,16,17]. These studies prompted our effort for developing a method based on another principle than on a simple comparison of retention times and capable to reveal at least partly the structure of separated analytes. Selection of standards for this study was based on the known metabolic pathways described for cortisol in humans.

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), 5β-pregnan-3β,11β,21-triol-20-one and 5α-pregnan-3α,11β,21-triol-20-one (allotetrahydrocorticosterone) were purchased from Sigma (St. Louis, MO, USA), pregn-4-ene-6β,11β,21-triol-3,20-dione (6β-hydroxycorticosterone), pregn-4-ene-20β,21-diol-3,11-dione, 5α-pregnan-3β,11β,21-triol-20-one, pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone), 5β-androstan-3α,11β-diol-17-one, 5α-androstan-3α,11β-diol-17-one, 5β-androstan-3α-ol-11,17-dione, 5α-androstan-3α-ol-11,17-dione, 5α-pregnan-3α,21-diol-11,20-dione, 5β-pregnan-3α,21-diol-11,20-dione (tetrahydro-11-dehydrocorticosterone), 5β-pregnan-3α,11β,20β,21-tetrol and 5β-pregnan-3α,20β,21-triol-11-one were from Steraloids (Wilton, NH, USA). Methanol was HPLC-gradient grade from Merck (Darmstadt, Germany); Milli-Q water (Millipore, Bedford, MA, USA) was used throughout this study.

2.2. Instrumentation

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

2.3. Chromatography

Chromatographic separation was carried out on the Zorbax Eclipse XDB C_{18} column (150×2.1 mm I.D., 5 μm, Rockland Technologies [Hewlett-Packard]). A 5-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–water–acetic acid, 60:40:1, v/v/v). Gradient started from 10% B to 40% B at 30 min, followed by a 20 min gradient to 50% B; then the column was eluted with 100% B for 5 min. Equilibration before the next run was achieved by 10 min washing with buffer A. Flow-rate was 0.250 ml/min, column temperature was held at 25°C and UV absorbance detection was done at 245 nm.

2.4. API–ESI–MS

API–ESI positive mode MS was used. Operating conditions were optimized by flow injection analysis (FIA) and were determined as: drying gas (N_2), 6 l/min; drying gas temperature, 350°C; nebulizer pressure, 20 p.s.i. (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. Assay of corticosterone metabolites in avian intestine

Incubation of the intestinal tissue (hen jejunum) with corticosterone or 11-dehydrocorticosterone was made according Vylitová et al. [6]. Briefly, corticosterone or 11-dehydrocorticosterone (1.45 μ*M*) was incubated with the tissue slices (300 mg) in sealed vessels containing 10 ml of oxygenated incubation solution (in m*M*): 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. The incubation medium was centrifuged at 3000 *g* for 10 min. The pellet was discarded and the supernatant was passed through the Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). Prior to use the cartridge was conditioned by passing through 5 ml of methanol and 5 ml of water. 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 100 μl methanol with 1% acetic acid for analysis.

3. Results and discussion

3.1. Separation of standards

Only five compounds of the steroid family are accessible by UV detection after their chromatographic separation (pregn-4-ene-6β,11β,21-triol-3,20-dione, pregn-4-ene-21-ol-3,11,20-trione, pregn-4-ene-20β,21-diol-3,11-dione, pregn-4-ene-11β,21-diol-3,20-dione, pregn-4-ene-11β,20β,21-triol-3-one); MS enables detection of all steroids investigated (Fig. 1) and is applicable to a much wider set of compounds. In Fig. 1A the absorbance peak emerging at 49 min is an impurity based on a slight difference in retention time and spectral data (the steroid involved should be devoid of UV absorbance). The applied chromatographic method, in its optimized version is uncomplicated and exploits a simple methanol–water elution system. However, the separation of compounds is not ideal, particularly for peaks eluted between 33 and 38 min. Mass spectra of individual standards are shown in Fig. 2. Selection of characteristic ions for extracted ion analysis allowed to identify and quantify all investigated metabolites (Fig. 3). As shown in Fig. 3 good separation was obtained for compounds with the same molecular mass or characteristic ions, typically α and β isomers of two compounds (Nos. 8 and 9; 10 and 11; 13 and 14) and three compounds (No. 5, 6 and 12). These results offered the possibility to use the present method for both qualitative and quantitative analysis.

Calibration curves (based on peak areas of selected extracted ions) were linear in the region 2–60 ng (five points, six repeated measurements, 2.3, 4.7, 11.75, 29.5 and 59.0 ng of standards injected). The correlation coefficients were larger than 0.986 with a precision (expressed as coefficient of variation, CV) within 10%. Accuracy for spiked samples (recovery) fell within the 85.7–95.3%. Some typical examples of calibration curves ($y=a+bx$, where y is peak area and x is amount of steroid) are: for 11-dehydrocorticosterone $a=120.10^3\pm25.10^3$, $b=220.10^3\pm6.10^3$, $r^2=0.9960$, and for corticosterone $a=112.10^3\pm28.10^3$, $b=259.10^3\pm6.10^3$, $r^2=0.9988$.

Detection limits (LODs) (signal-to-noise ratio 3:1) of the signal of the extracted ions are summarized in Table 1 and refer to spiked samples. These limits are compatible with data reported by Shibasaki et al. [7] (1–0.25 ng) and Fiori et al. [10] (5–2 ng) for other steroids.

ESI–MS spectra were characterized by the quasi-molecular ions $[M+H]^+$ (except for compound 15), accompanied by the $[M+H-18]^+$ and $[M+H-36]^+$ fragment ions, resulting from the loss of one and two water molecules respectively. Ions $[M+23]^+$ and $[M+39]^+$ are probably adduct ions corresponding to $[M+Na]^+$ and $[M+K]^+$. This finding is in concurrence with results reported by Shibasaki et al. [7] with thermospray and Fiori et al. [10] with APCI for various corticosteroids. On the contrary, ion $[MH-60]^+$ derived by loss of glycoaldehyde was not observed. Similar results were reported by Wiedmer et al. [15] for MECC–ESI–MS of four corticosteroids (cortisone, cortisol, corticosterone and 11-dehydroaldosterone).

Admittedly, ESI is probably not the best atmospheric pressure ionization technique for corticosteroids owing to quasi-molecular ion adducts by

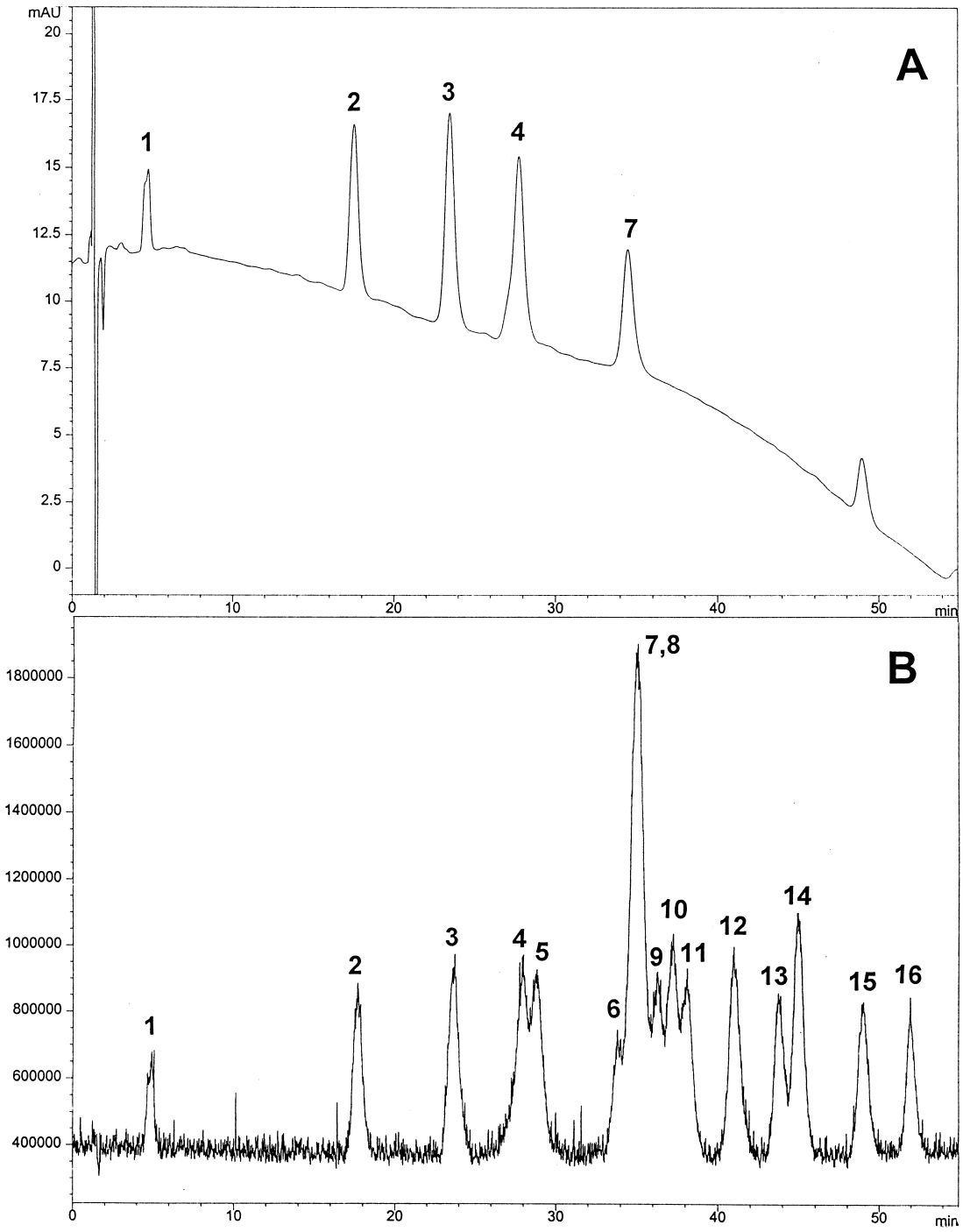

Fig. 1. Separation of standard mixture of corticosteroid metabolites monitored by (A) UV absorbance detection at 254 nm and (B) mass detection (TIC 200–500 m/z). For conditions see Experimental; injection, 59 ng of each steroid. For structure identification see Fig. 2.

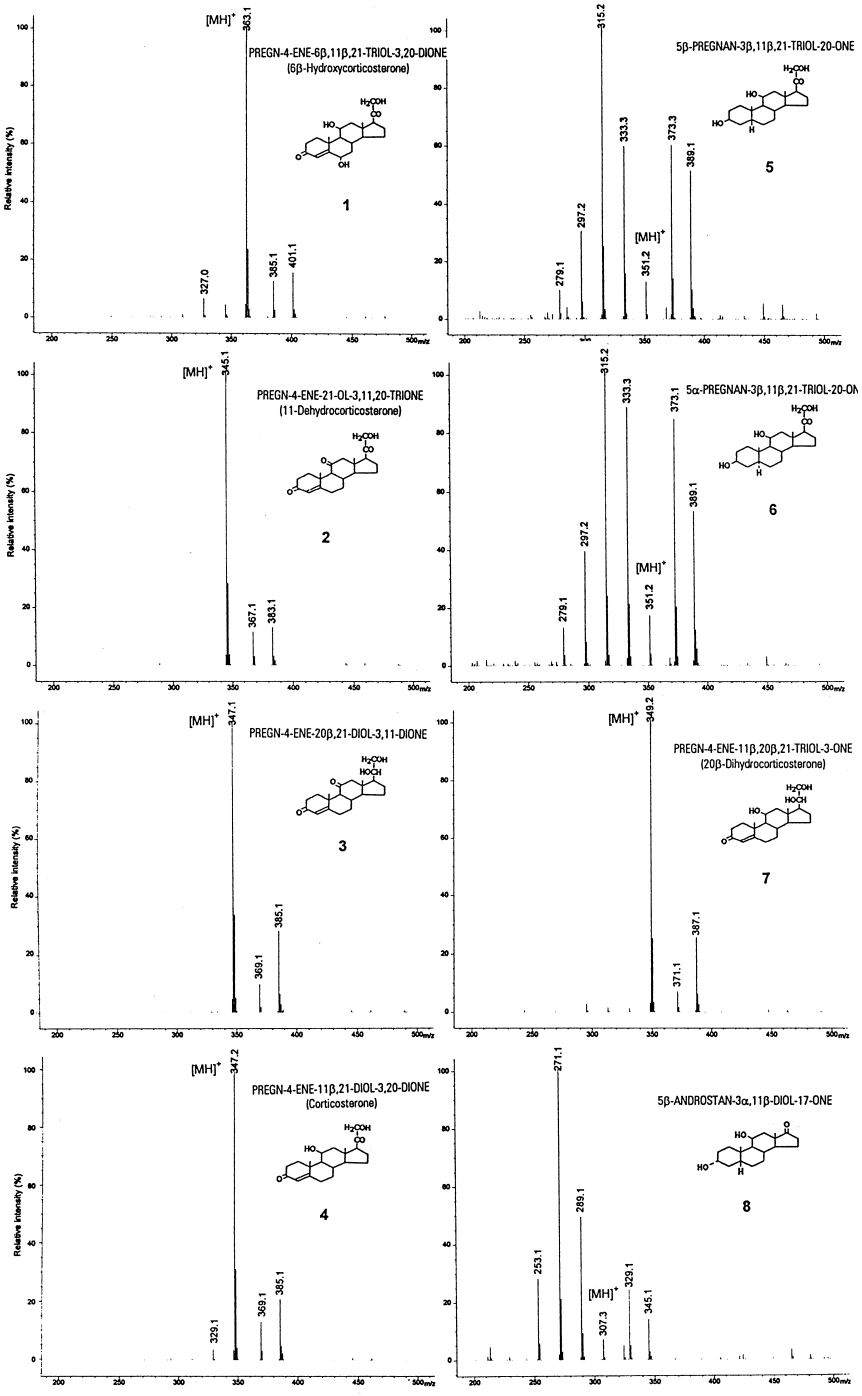

Fig. 2. API–ESI mass spectra of corticosteroid metabolite standards, numbers in parentheses mean order in HPLC separation.

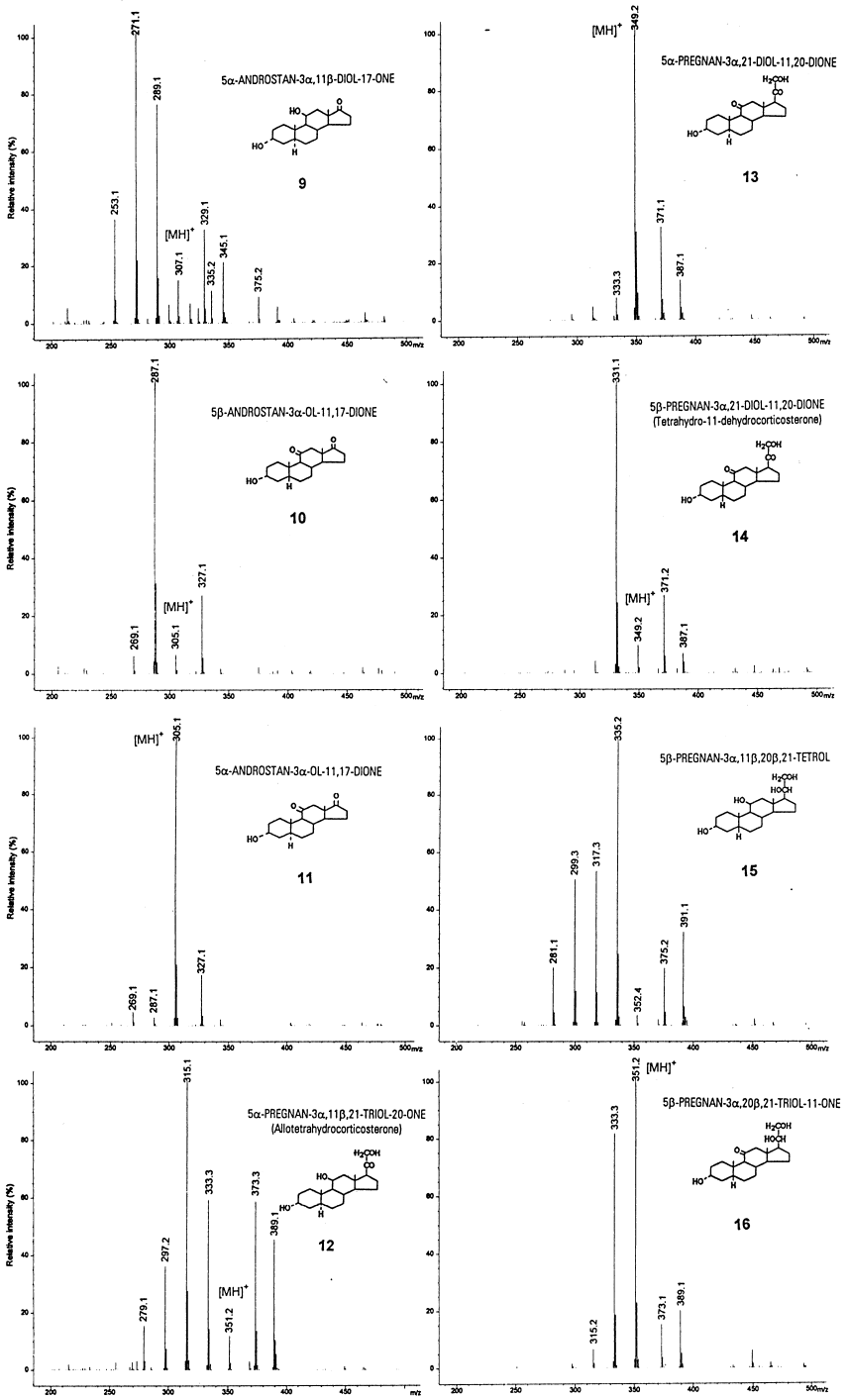

Fig. 2. (*continued*)

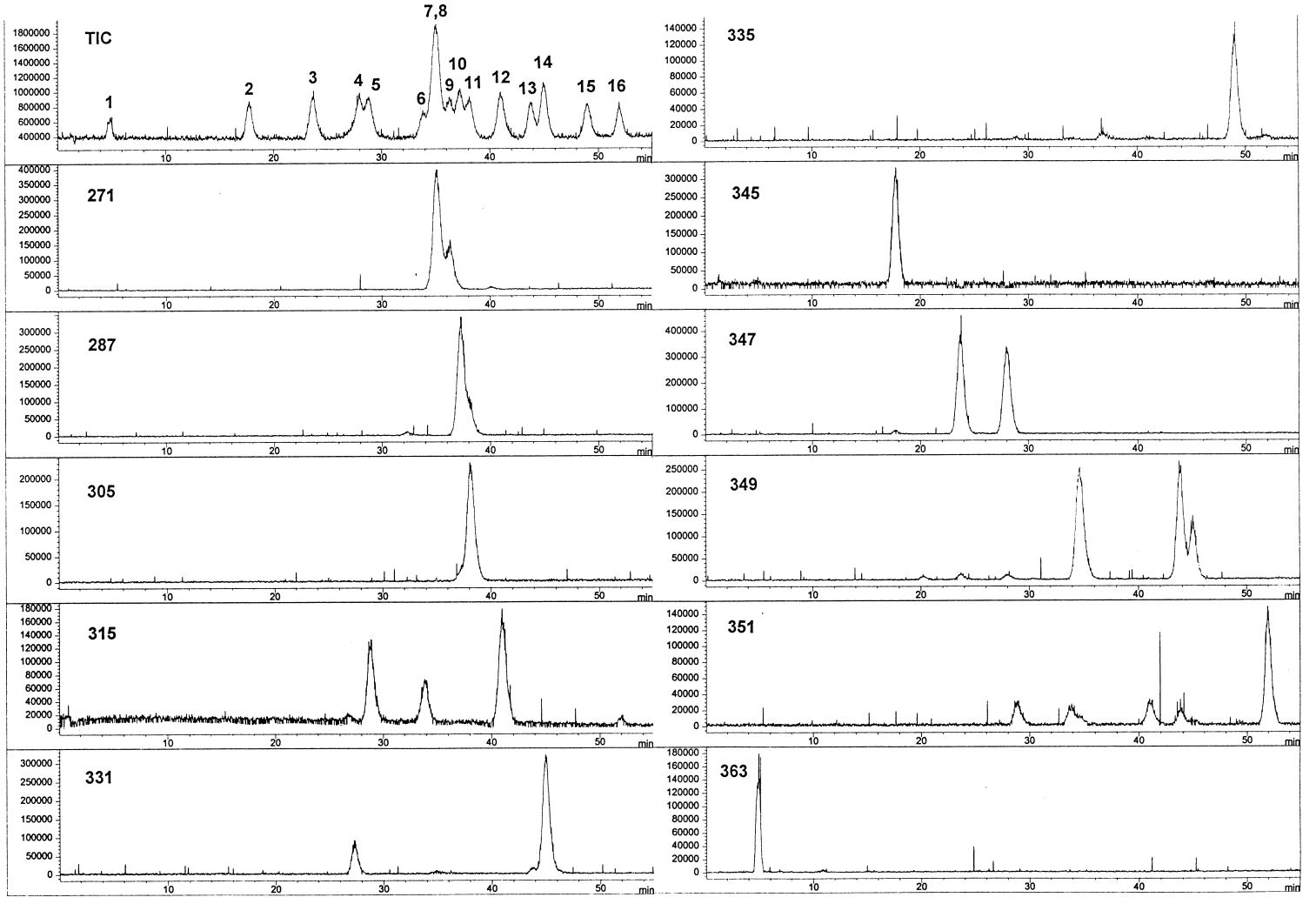

Fig. 3. The same separation of steroids as in Fig. 1, monitored by extracted ions. Specification of individual ions (m/z): **271**, 5β-androstan-3α,11β-diol-17-one (**8**) and 5α-androstan-3α,11β-diol-17-one (**9**); **287**, 5β-androstan-3α-ol-11,17-dione (**10**); **305**, 5α-androstan-3α-ol-11,17-dione (**11**); **315**, 5β-pregnan-3β,11β,21-triol-20-one (**5**), 5α-pregnan-3β,11β,21-triol-20-one (**6**) and 5α-pregnan-3α,11β,21-triol-20-one (allotetrahydrocorticosterone) (**12**); **331**, 5β-pregnan-3α,21-diol-11,20-dione (tetrahydro-11-dehydrocorticosterone) (**14**); **335**, 5β-pregnan-3α,11β,20β,21-tetrol (**15**); **345**, pregn-4-ene-21-ol-3,11,20-trione (11-dehydrocorticosterone) (**2**); **347**, pregn-4-ene-20β,21-diol-3,11-dione (**3**), pregn-4-ene-11β,21-diol-3,20-dione (corticosterone) (**4**); **349**, pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone) (**7**) and 5α-pregnan-3α,21-diol-11,20-dione (**13**); **351**, 5β-pregnan-3α,20β,21-triol-11-one (**16**); **363**, pregn-4-ene-6β,11β,21-triol-3,20-dione (6β-hydroxycorticosterone (**1**).

Table 1
Peak identification, extracted ions used for quantification and detection limits (LODs) (signal-to-noise ratio 3:1) of the signal of the extracted ions

Peak No.	Compound	Extracted ion (m/z)	LOD (ng)
1	Pregn-4-ene-6β,11β,21-triol-3,20-dione (6β-hydroxycorticosterone)	363	1.0
2	Pregn-4-ene-21-ol-3,11,20-trione (11-dehydrocorticosterone)	345	0.5
3	Pregn-4-ene-20β,21-diol-3,11-dione	347	0.5
4	Pregn-4-ene-11β,21-diol-3,20-dione (corticosterone)	347	0.5
5	5β-Pregnan-3β,11β,21-triol-20-one	315	1.0
6	5α-Pregnan-3β,11β,21-triol-20-one	315	2.0
7	Pregn-4-ene-11β,20β,21-triol-3-one (20β-dihydrocorticosterone)	349	0.5
8	5β-Androstan-3α,11β-diol-17-one	271	0.5
9	5α-Androstan-3α,11β-diol-17-one	271	1.0
10	5β-Androstan-3α-ol-11,17-dione	287	0.5
11	5α-Androstan-3α-ol-11,17-dione	305	0.5
12	5α-Pregnan-3α,11β,21-triol-20-one (allotetrahydrocorticosterone)	315	1.0
13	5α-Pregnan-3α,21-diol-11,20-dione	349	0.5
14	5β-Pregnan-3α,21-diol-11,20-dione (tetrahydro-11-dehydrocorticosterone)	331	0.5
15	5β-Pregnan-3α,11β,20β,21-tetrol	335	0.5
16	5β-Pregnan-3α,20β,21-triol-11-one	351	1.0

cationization (see Fig. 2). APCI is preferable as these adduct ions are absent in the spectra leading to more abundant molecular ion $[M+H]^+$ or $[M-H]^-$ depending on whether the positive or negative mode is used [18]. In practice, however also ESI can be used for naturally occurring samples as demonstrated by Dodds et al. [19], who were able to assay cortisol in placental perfusate by this approach.

3.2. Applicability

Applicability of the present method was verified by monitoring corticosterone metabolism in hen intestine. As previously described by our group [6], in avian metabolism the presence of 20β-hydroxysteroid dehydrogenase has been suspected (besides 11β-hydroxysteroid dehydrogenase). The former enzyme converts corticosterone to the 20β-dihydrocorticosterone and 11-dehydrocorticosterone to the 11-dehydro-20β-dihydrocorticosterone. This conclusion was derived from the comparison of retention times of the respective steroids obtained by HPLC and micellar electrokinetic chromatography with diode-array detection [6]. The results obtained by HPLC–API–ESI–MS supported the hypothesis about the presence of 20β-hydroxysteroid dehydrogenase activity in hen's intestine (Fig. 4): corticosterone was converted to the 20β-dihydrocorticosterone and 11-dehydrocorticosterone while 11-dehydrocorticosterone was the parent compound for 11-dehydro-20β-dihydrocortocosterone and 20β-dihydrocorticosterone. This finding accords to the previously described activity of these two enzymes (20β-hydroxysteroid dehydrogenase and 11β-hydroxysteroid dehydrogenase) [6]. Mass spectra of the metabolites seen in the incubation mixture fit well to the spectra of the standards (Fig. 5). A finding that should be stressed is the presence of 5β-pregnan-3α,20β,21-triol-11-one in the above mixtures, particularly in the incubation mixture containing 11-dehydrocorticosterone and is worth further attention. Other peaks resolved

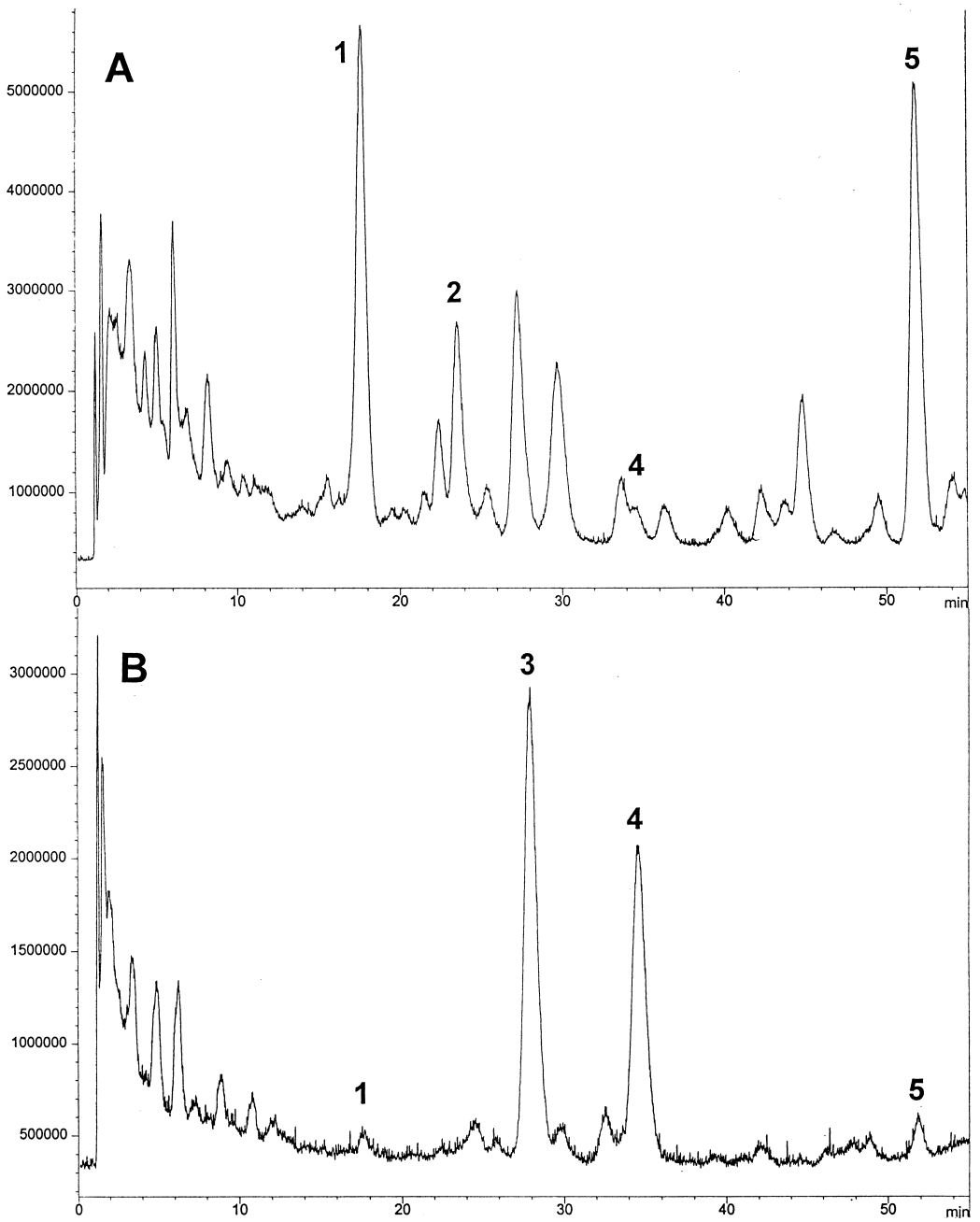

Fig. 4. HPLC–API–ESI–MS profile (TIC) of incubation of the hen jejunum with (A) corticosterone or (B) 11-dehydrocorticosterone. Peaks: 1=11-dehydrocorticosterone (pregn-4-ene-21-ol-3,11,20-trione); 2=11-dehydro-20β-dihydrocorticosterone (pregn-4-ene-20β,21-diol-3,11-dione); 3=corticosterone (pregn-4-ene-11β,21-diol-3,20-dione); 4=20β-dihydrocorticosterone (pregn-4-ene-11β,20β,21-triol-3-one); 5=5β-pregnan-3α,20β,21-triol-11-one. For conditions see Experimental.

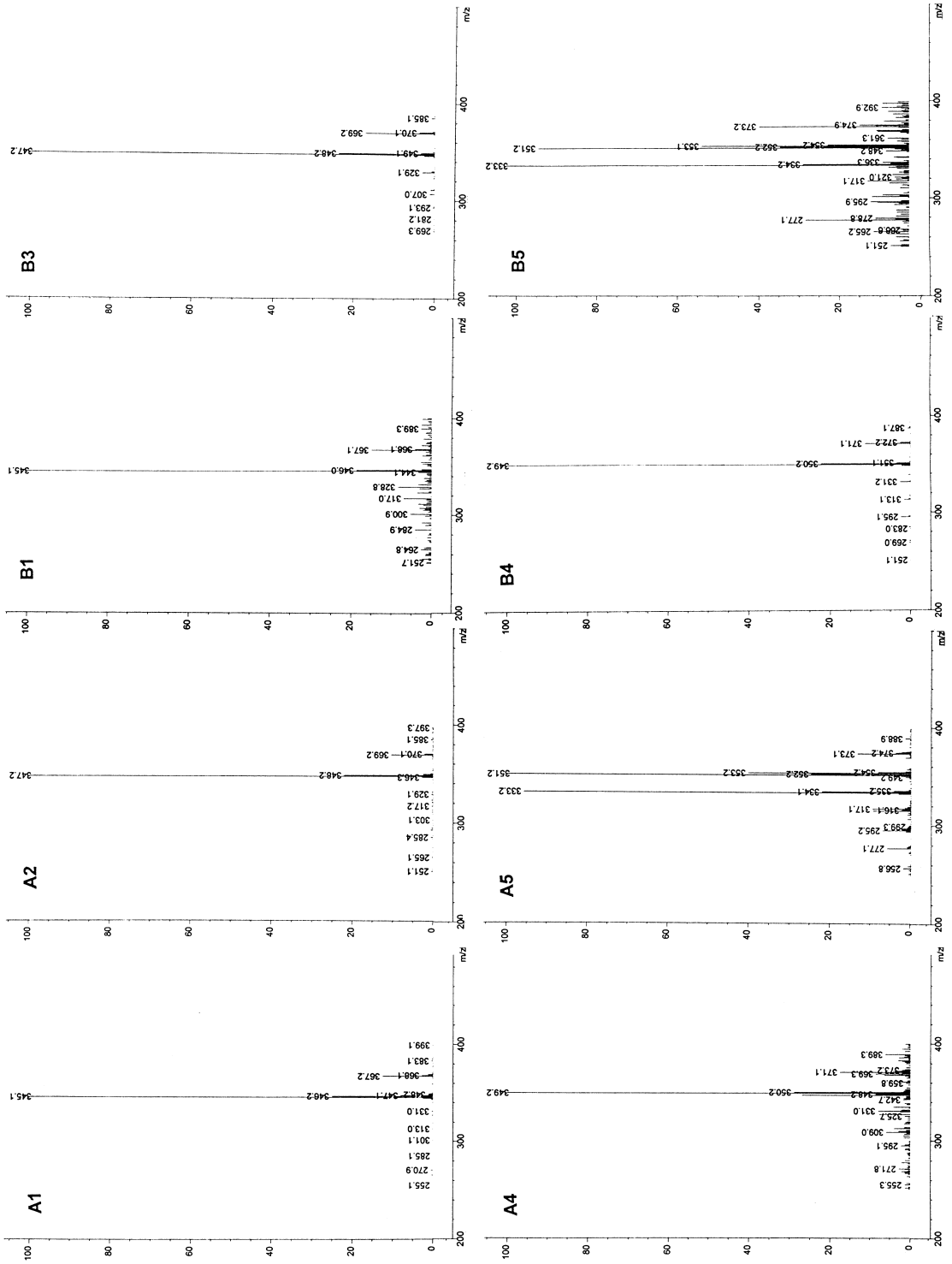

Fig. 5. API–ESI mass spectra of compounds from Fig. 4.

by HPLC do not correspond to any of the steroid standards which may lead to the conclusion that they either do not stem from corticosteroid metabolism or represent unpredicted metabolic products arising from the specific nature of corticosteroid metabolism in birds.

In conclusion, it can be stated that the separation and identification procedure described is applicable to the glucocorticoid metabolism assay in the avian intestine where activities of both 11β-hydroxysteroid and 20β-hydroxysteroid dehydrogenases were revealed.

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