Separation and Determination of Synthetic Corticosteroids in Bovine Liver by LC-Ion-Trap-MS-MS on Porous Graphite

C. Baiocchi*1 / M. Brussino1 / M. Pazzi1 / C. Medana1 / C. Marini2 / E. Genta2

1 Dipartimento di Chimica Analitica, Università di Torino, via P. Giuria 5, 10125 Torino, Italy; E-Mail: claudio.baiocchi@unito.it
2 Marini Group Laboratory, C.so Francia 227g, 10098 Rivoli (TO), Italy

Key Words
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Summary
A method is presented for the separation and detection of eight synthetic corticosteroids in bovine liver tissues. Following sample preparation, the samples were separated by isocratic LC (mobile phase: 85% methanol, 15% dichloromethane) using a porous graphite (PGC) column and detected by ion trap MS-MS, using an atmospheric pressure chemical ionization (APCI) source and monitoring positive ions. An octadecyl-silica phase system was also used for comparison. With the PGC stationary phase the critical isomer separation of dexamethasone and betamethasone was achieved. The performances of MS-MS, MS3, selected ion monitoring (SIM) and selected reaction monitoring (SRM) detection modes were checked and best conditions of sensitivity were obtained in MS-MS mode.

Introduction
Several growth promoting substances (natural and synthetic hormones) are circulating in legal and illegal markets and are frequently used in cattle fattening. Such xenobiotic compounds are orally administered as they gave no injection sites. However to avoid their detection in the liver of slaughtered animals during periodical controls it is common practice to interrupt their administration some weeks before slaughtering. In this case however the cattle rapidly lose weight and the quality of their meat deteriorates. As is well-known the synthetic corticosteroids, which are widely used for the treatment of inflammation allergies and deseases related to adrenal cortex insufficiency, possess the property of prolonging the effect of growth-promoting substances after interruption of administration, so they are used as anabolic-like substances during the last weeks of life of cattle. Much more effective are dexamethasone and betamethasone but sometimes prednisone, prednisolone, flumethasone, triamcinolone, triamcinolone acetonide or flunisolide may be used alternatively (Figure 1).

The aim of the present study was to develop screening and confirmatory methods to support monitoring of the eight cited substances, optimised both as regards selectivity and sensitivity. Mass spectrometry is the only detection technique able to assure such requirements by virtue of its ability to provide unambiguous determination of analyte structure and detector signals of high selectivity and intensity. However, in some cases fluorometric determination is proposed after suitable derivatization [1].

GC-MS has been mainly used in the past for the analysis of these compounds [2] until the recent development of LC-MS techniques. Both thermospray [3] and ion spray techniques [4] gave good results in terms of selectivity and sensitivity even if the best results were obtained by atmospheric-pressure chemical-ionization (APCI) [5]. The more comprehensive of these methods was a confirmatory HPLC-MS-MS method for ten synthetic corticosteroids in bovine urines [6] where a triple quadrupole with an APCI ionization source in negative ion mode was used. In recent work several LC separations of corticosteroid residues with APCI-MS detection were on both equine urine [7] and on bovine liver [8], but using RP stationary phases the critical isomer separation dexamethasone-betamethasone was achieved only after long elution times.

In the present study, in order to optimise the selectivity of the chromatographic separation, a porous graphite column [9] was used to exploit its peculiar separation mechanism based, among other things, on the planarity properties of molecules and mainly to separate the typical critical pair of isomers: dexamethasone and betamethasone. An octadecyl-silica...
phase system was also used for comparison purposes. Mobile phases of different selectivity were checked and their compatibility with the ionization mechanism of APCI ion source were compared.

The positive ion mode of operation was adopted and different detection conditions were tested (SIM, MS/MS, SRM) to optimise sensitivity.

The treatment of real samples (liver from slaughtered animals) was designed with the aim of reducing the number of operative steps as much as possible—consistent with a good recovery factor.

**Experimental**

**Chemicals**

HPLC grade water was from MilliQ Academic system (Millipore, Milan, Italy). Methanol and acetonitrile HPLC grade (Merck Eurolab, Milan, Italy) were filtered through a 0.45 μm filter before use. Ammonium acetate and the eight standard corticosteroids were from Sigma-Aldrich, Milan, Italy. Standard solutions in methanol, 5 mg mL⁻¹, of the corticosteroids were prepared monthly and stored at −20 °C. Standard solutions at lower concentrations were prepared daily in the mobile phase.

**Liquid Chromatography**

LC analysis was on a TSP system (AS300 autosampler, P4000 pump, Thermo Finnigan, Milan, Italy). Chromatographic conditions were: a) porous graphitic carbon column (Hypercarb 5 μm, 125 × 4.6 mm, Thermo Hypersil, Milan, Italy): isocratic elution, 85% methanol, 15% dichloromethane, flow 1.0 mL min⁻¹. b) C18 column (Inertsil 5 ODS-3, 250 × 4.6 mm, Varian-Chrompack, Leini (TO), Italy): isocratic elution, 65% methanol, 35% aqueous ammonium acetate 20 mM, pH 6.8, flow 1.0 mL min⁻¹. Injection volumes of 100–200 μL were used. Typical chromatograms are shown in Figure 2.

**Mass Spectrometry**

A LCQ ion-trap MS (Thermo Finnigan, Milan, Italy) equipped with an atmospheric pressure ion source and APCI interface was used. The LC column effluent was delivered to the ion source through a heated nebulizer probe (400 °C) using nitrogen as sheath and auxiliary gas (Claинд nitrogen generator apparatus). The corona discharge voltage was set at 5 kV. The heated capillary temperature was maintained at 300 °C to minimise the presence of interfering m/z values due to clusters of solvent molecules with electrolyte ions. Positive ions were acquired in single ion monitoring (SIM), MS-MS and SRM with the aim of comparing the respective sensitivities.

The acquisition method used was optimised by tuning sections for each substance (capillary, magnetic lenses and collimating octapole voltages) to achieve maximum sensitivity.

**Sample preparation**

2.5 g liver were added to 5.0 mL ammonium acetate 0.05 M, pH 6.8 and sonicated in an ultrasonic bath at 30% of maximum power (50 W) for 10 min, followed by centrifugation at 2500 g for 5 min. The decanted supernatant was lyophilised and the dry residue was recovered with 500 μL of 50: 50 methanol-ammonium acetate 20 mM.

**Results and Discussion**

**Extraction and LC-MS-MS**

The procedure used for the extraction of the eight corticosteroids is very simple, having only one extraction step and minimum handling of the sample.

To detect each corticosteroid with maximum sensitivity and at the same time of selectivity tuning of the detector response was made on each characteristic (M+1)⁺ ion and subsequently their fragmentation behaviour was investigated acquiring the respective MS-MS spectra in collision activated dissociation mode (CAD). Direct infusion of solutions of pure standards in the API source by pump syringe made this procedure easy and straightforward.

Two example MS-MS spectra are reported in Figure 3 and the major common fragmentation pathways in Table I.

As can be seen the fluorinated corticosteroids (dexamethasone, betamethasone, flumethasone, flunisolide, triamcinolone and triamcinolone acetonide) lost primarily the HF molecule (20 Da) and subsequently molecules of H₂O (18 Da). The non-fluorinated substances prednisone and prednisolone lost H₂O and CO (28 Da) molecules.

Once the fragmentation behaviour of the eight analytes was known it was possible to make the analysis in MS-MS detection mode which, as expected, exhibited better detection sensitivity and selectivity than full MS mode.