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Detection of exogenous hydrocortisone in horse urine by gas chromatography–combustion–carbon isotope ratio mass spectrometry

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Abstract

A gas chromatography–combustion–isotope ratio mass spectrometry method for confirmation of hydrocortisone abuse in horseracing and equine sports is proposed. Urinary hydrocortisone was converted to a bismethylenedioxy derivative which presents good gas chromatographic properties and brings an extra carbon contribution of only two carbon atoms. Synthetic hydrocortisone has a different ^{13}C abundance from that of natural urinary horse hydrocortisone and the difference is significant, therefore exogenous and endogenous hydrocortisone can be distinguished. © 1997 Elsevier Science B.V.

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1. Introduction

Corticosteroids can be administered to race horses to improve their performance. They are used primarily as anti-inflammatory drugs that also relieve pain and fatigue. The administration of either naturally occurring or synthetic corticosteroids is banned in horseracing and equine sports. Doping control is performed routinely on urine or blood samples by specialized laboratories.

The presence of xenobiotic compounds such as synthetic corticosteroids can be detected relatively easily by immunoassay and liquid chromatography screening methods [1]. Confirmation of such findings is now carried out by LC–MS [2,3].

Naturally occurring corticosteroids, hydrocortisone (cortisol) and cortisone are easy to detect, but their origin, endogenous or exogenous, cannot be determined by gas chromatography–mass spectrometry. The Fédération Internationale des Activités Hippiques has established a cut-off for hydrocortisone at 1 μg/ml in horse urine [4], above which the laboratory must quantitate the compound and report the sample as positive. Unfortunately, it is possible

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to administer hydrocortisone to a horse while keeping the urinary hydrocortisone concentration below that cut-off. If carbon isotope ratio mass spectrometry makes it possible to distinguish exogenous from endogenous hydrocortisone, it could help resolve such cases, and all cases in which hydrocortisone administration is suspected.

All carbon in biomolecules is ultimately derived from atmospheric CO_2 fixed by photosynthesis by two main pathways. During this process there is a discrimination in the ^{13}C isotope uptake. In animals the ^{13}C content reflects an average of the ^{13}C content of all the plant material eaten, when synthetic hydrocortisone is generally made from a single plant species (soya) with a defined ^{13}C content.

The connection of a gas chromatograph to an isotope ratio mass spectrometer via a combustion interface allows the calculation of the ratios of the stable isotopes of carbon: ^{13}C and ^{12}C by the measurements of the isotopomers of CO_2 from all the carbon atoms in the molecule of interest.

Previous studies have shown that differentiation between natural human testosterone and synthetic testosterone is possible by measuring the $^{13}C/^{12}C$ ratio [5–7].

This paper presents a preliminary evaluation of gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) to differentiate natural endogenous horse hydrocortisone from pharmaceutical hydrocortisone by carbon isotope ratio measurements.

2. Experimental

2.1. Chemicals and reagents

All solvents and reagents were of analytical-grade purity. Hydrocortisone reference compounds were purchased from Sigma-Aldrich Chimie (St. Quentin Fallavier, France) and Steraloids (Interchim, France). Pharmaceutical hydrocortisone, hydrocortisone Roussel, was obtained from Laboratoires Roussel (Romainville, France). [9,12,12-^{2}H]Hydrocortisone was purchased from Euriso-Top (CEA, Saclay, France). Paraformaldehyde (powder, 95%) was from Aldrich (St. Quentin Fallavier, France) Chem Elut columns CE 1010 (Kieselguhr) were obtained from Varian (Les Ulis, France).

2.2. Urine samples

The urine samples are described in Table 1. Three experimental horses, A, B and C, were selected for hydrocortisone administration. Urine numbers 1–3, are blank urines collected 2 days before hydrocortisone administration from horses A, B and C, respectively. Urine numbers 4–7 were collected after intravenous administration of hydrocortisone (1 mg/kg) to the three horses. Urines 4 and 5 were taken from horses A and B, respectively, and urines 6 and 7 were taken from horse C, 30 min and 2 h after administration. Urine numbers 8–13 are post-race urines collected during routine control, i.e. less than 1.5 h after the race.

2.3. Hydrocortisone extraction

Two 10-ml urine samples were adjusted to pH 9.5 with diluted aqueous ammonia (98:2, v/v). The solution was poured through a Chem Elut CE 1010 column. Hydrocortisone was eluted with 50 ml of a dichloromethane–ethanol mixture (99:1, v/v) and the solvent evaporated to dryness.

2.4. Hydrocortisone quantification

Hydrocortisone was quantified in the samples according to the previously described LC–APCI–MS method [8]. Quantification was performed in the selected ion monitoring mode using m/z 363 for hydrocortisone, m/z 365 for 20β-dihydrocortisol and m/z 366 for deuterium-labelled hydrocortisone. Standard curves were established over the range of 0–300 ng/ml of hydrocortisone. Quality control samples were prepared in blank urine. The blank urine was obtained from a horse which had received two doses of dexamethasone phosphate 12 h apart to suppress endogenous hydrocortisone production. Reproducibility (inter-day variation) was determined over a 12-day period ($n=3$) at concentrations of 50, 150 and 300 ng/ml of hydrocortisone. The coefficients of variation were 5.7, 4.9 and 3.5%, respectively. The hydrocortisone limit of quantification was

Table 1
Horse urine samples

Horse	Urine	Type	Urine (volume/ml)	Hydrocortisone (ng/ml)
A	1	Baseline	4×20	27
B	2	Baseline	4×20	25
C	3	Baseline	4×20	26
A	4	Treated[a,b]	20	4951[g]
B	5	Treated[a,c]	20	13 560[g]
C	6	Treated[a,d]	20	7023[g]
C	7	Treated[a,e]	20	12 295[g]
D	8	Unknown[f]	20	317
E	9	Unknown[f]	20	315
F	10	Unknown[f]	20	271
G	11	Unknown[f]	20	212
H	12	Unknown[f]	20	221
I	13	Unknown[f]	20	219

[a]Treated: intravenous (i.v.) injection of 500 mg of hydrocortisone Roussel® (~1 mg/kg).
[b]Treated: urine collected 30 min after i.v. injection.
[c]Treated: urine collected 50 min after i.v. injection.
[d]Treated: urine collected 30 min after i.v. injection.
[e]Treated: urine collected 2 h after i.v. injection.
[f]Unknown: normal post-race urine sample.
[g][Hydrocortisone]>1 μg/ml: positive.

20 ng/ml, the limit of detection was 5 ng/ml and reproducibility was 5% at 20 ng/ml.

2.5. Purification

Hydrocortisone purification was carried out by semi-preparative liquid chromatography on an HP-1050 (Hewlett-Packard) instrument. The hydrocortisone extract was redissolved in 110 μl of methanol and injected onto a Spherisorb ODS2 (25×1 cm I.D., 5 μm; Interchim) via a 100-μl sample loop injection. The mobile phase was an acetonitrile–water (35:65, v/v) mixture delivered at 2.8 ml/min. Elution was isocratic and was monitored by UV absorbance detection (Kratos Spectroflow 757, Manchester, UK) at 240 nm. The hydrocortisone fraction was collected in the 10.5–12.5 min time range.

2.6. Derivatization

The dried hydrocortisone fraction from LC was redissolved in 200 μl of dichloromethane and then treated with 500 μl of acidified *p*-formaldehyde which was freshly prepared by stirring a mixture of *p*-formaldehyde (1 g), water (3 ml) and concentrated hydrochloric acid (3 ml) at room temperature until the suspension became homogeneous. The resulting mixture was vigorously stirred (Vortex) at room temperature for 30 min, and the aqueous layer was separated. The organic layer was diluted with dichloromethane (500 μl) and then washed successively with saturated aqueous sodium bicarbonate and water (1×500 μl). The organic layer containing the bismethylenedioxy derivative (BMD) was separated and evaporated to dryness under a nitrogen stream.

2.7. GC–EI-MS

The hydrocortisone bismethylenedioxy derivative (hydrocortisone BMD) was redissolved in 0.1 or 1 ml of dichloromethane depending on urinary hydrocortisone concentration.

Hydrocortisone BMD analyses were performed on HP-5890 (Hewlett-Packard) gas chromatograph equipped with a SGE BPX5 (95% methyl, 5% phenyl silicone) fused-silica capillary column (25 m×0.2 mm I.D., 0.25 μm film thickness) linked to an HP-5970 B mass selective detector. The injections (1 μl) were made in the splitless mode using an HP-7673 auto-injector. The carrier gas was helium (head pressure 100 kPa). The initial oven tempera-

ture was set at 280°C and, after 1 min, it was raised to 320°C at 6°C/min. The final oven temperature was maintained for 14 min. The injector and transfer line temperatures were set at 280°C.

2.8. Isotopic analysis

GC–C–IRMS analyses were performed on an HP-5890 gas chromatograph connected to a laboratory-designed combustion furnace (Service Central d'Analyse, CNRS, Vernaison, France) linked to a Finnigan MAT 252 isotope ratio mass spectrometer. The GC was equipped with an ULTRA-2 column (Hewlett-Packard, 5% phenyl, 95% methylsilicone, 25 m×0.32 mm I.D., 0.17 μm film thickness). Helium was the carrier gas (170 kPa). Samples were injected in the splitless mode (1 μl) at 280°C. The GC temperature program was set at 150°C for 1 min, then the oven temperature was increased at 7°C/min up to 315°C and maintained at the final temperature for 5 min. The temperature of the combustion furnace was set at 800°C and the oxidative catalyst consisted of two copper oxide wires (0.10 mm) and a platinum wire (0.10 mm) in quartz tubing of 0.5 mm in diameter.

The δ standard notation for expressing carbon isotope ratios is defined as the relative difference in isotope ratio between the sample and an international standard (PDB, Pee Dee Belemnite), calculated as:

$$\delta^{13}\text{C‰} = \frac{R_{\text{sample}} - R_{\text{PDB}}}{R_{\text{PDB}}} \times 1000$$

where R_x refers to the $^{13}C/^{12}C$ ratio of the sample or the international standard. Each sample was submitted to two or three measurements which were averaged. A typical standard deviation associated with these GC–C–IRMS determinations was ±0.5‰. Non-derivatized reference hydrocortisone samples from chemical suppliers were analyzed by direct introduction combustion/isotope ratio mass spectrometry on a Finnigan MAT Delta S isotope ratio mass spectrometer equipped with a source combustion interface. Accurately weighed samples (0.5–1 mg) were placed directly in the combustion furnace via a small silver basket.

3. Results and discussion

Our work was focused on the free urinary hydrocortisone as it is widely accepted as the target compound for horse doping control. Studies have shown that i.v. injection of hydrocortisone induces free hydrocortisone concentrations that are higher in urine than in blood [9]. Solid-phase extraction on kieselguhr is routinely used as the first step in urine sample preparation for corticosteroid screening. Changing the urine pH between 3 and 12 has no significant influence on recovery [10]. Adjustment to pH 9.5 with dilute aqueous ammonia was chosen because at that pH other illicit basic drugs are also extracted and can be detected if needed. The elution solvent was a mixture of dichloromethane and ethanol (99:1, v/v). The presence of ethanol improved recovery to 90–100% instead of ≈80% with dichloromethane [9].

Purification was achieved by semi-preparative LC on a C_{18} column. To ensure that excessive fractionation does not lead to artefactual enrichment in one or other isotope, the peak collection was wide enough to completely recover the ^{12}C and ^{13}C homologs which may get partially resolved [11]. Therefore during the hydrocortisone preparation we maximized recovery at the expense of purity to avoid any isotopic discrimination.

Preliminary assays of hydrocortisone by GC–C–IRMS were done without derivatization using a short column (10 m×0.25 mm I.D.) for GC. The results were not usable and interpretable because of a rapid decrease in sensitivity after three injections and signal disappearance after several more injections. Hydrocortisone must be derivatized to achieve good chromatographic properties. The derivatization of hydrocortisone is difficult, which is typical for a corticosteroid. The common derivative, trimethylsilyl (TMS), cannot be used because silica residues are not compatible with the combustion step of GC–C–IRMS, as it forms deposits on the copper wire and this inactivation leads to incomplete combustion. In addition, hydrocortisone gives a tris-TMS derivative with nine additional carbon atoms, which would substantially alter the isotope ratio of the 21 carbon atoms of native hydrocortisone.

The methoxime–bis-TMS derivative is more sta-

ble than the TMS derivative; it exists as a pair of *syn-anti* isomers that are generally resolved by GC. The large number of additional carbon atoms remains undesirable and a derivative that gives one, not two GC peaks, would be preferred.

The bismethylenedioxy derivative of hydrocortisone only has two additional carbon atoms. The reaction of hydrocortisone with *p*-formaldehyde forms a diacetal derivative at the 17-dihydroxyacetophenone side chain [12,13].

We carried out a kinetic study of the reaction in order to optimize the hydrocortisone derivatization yields. Derivatization was carried out at 30°C and room temperature, and for 30 min, 1, 1.5 and 2 h. The best derivatization yield was obtained at room temperature after 30 min.

The purity of each urinary hydrocortisone derivative was checked by GC–EI-MS. As described by Shibasaki et al. [12], the hydrocortisone BMD derivative afforded a molecular ion $M^{+\cdot}$ at *m/z* 404 and characteristic fragment ions at *m/z* 386 $[M-H_2O]^{+\cdot}$ and *m/z* 356 $[M-H_2O-HCHO]^{+\cdot}$ (Fig. 1).

Reference hydrocortisone compounds from different suppliers were first analyzed by direct introduction combustion–IRMS then by GC–C–IRMS of the corresponding BMD derivative. The results (Table 2) show that the derivation reaction slightly modifies the carbon isotope ratio measurements ($\Delta\delta$

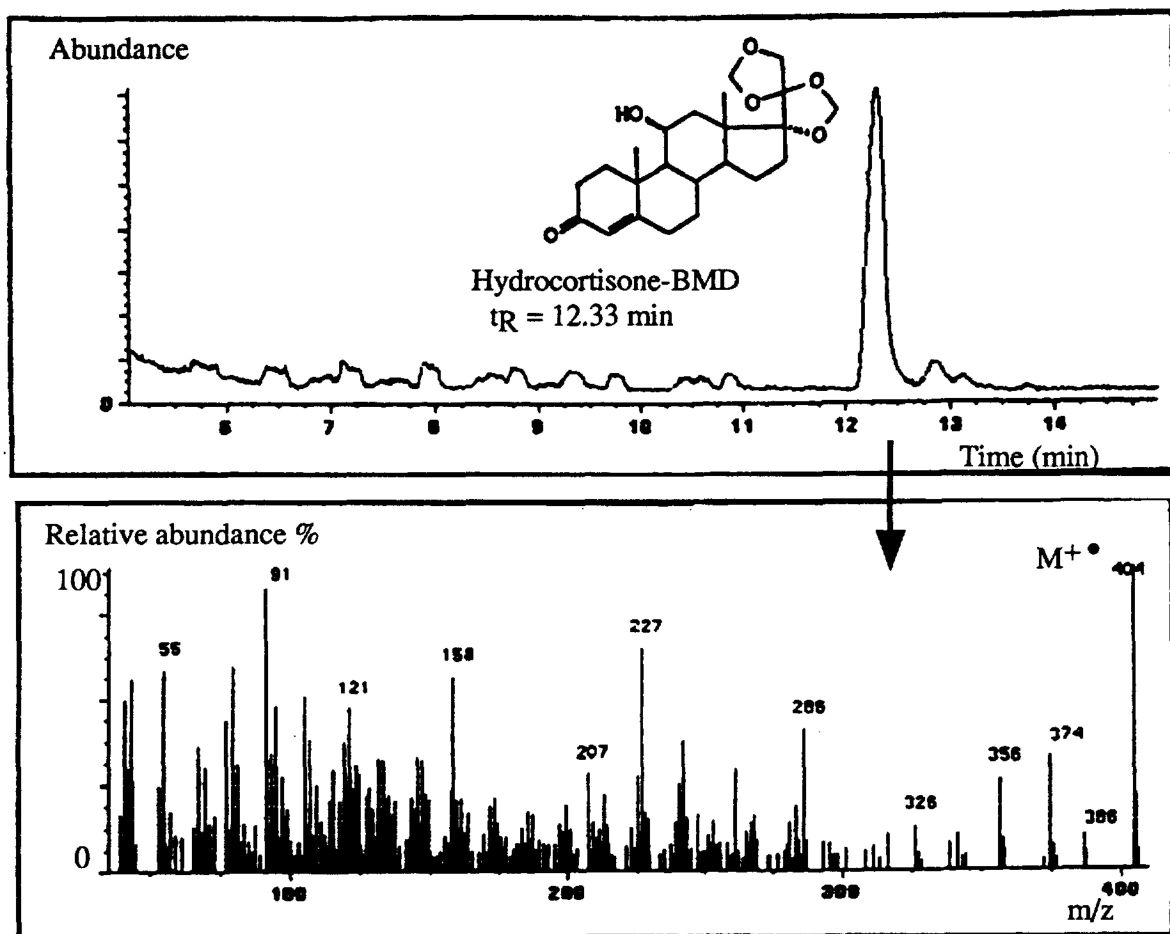

Fig. 1. GC–EI-MS analysis of hydrocortisone bismethylenedioxy derivative from post-race urine extract.

Table 2
Carbon δ‰ of chemicals and bismethylenedioxy derivatives (BMD derivatives)

Compound	δ‰ (C–IRMS) measured	δ‰ BMD derivative calculated[a]	δ‰ BMD derivative (GC–C–IRMS) measured	Δδ[b]
Hydrocortisone (Aldrich)	−30.3	−30.6	−31.1	1.5
Hydrocortisone (Sigma)	−29.4	−29.8	−31.4	1.6
Hydrocortisone (Steraloids)	−30.0	−30.4	−32.0	1.6
Hydrocortisone (Roussel®)	n.a.	n.a.	−31.0	n.a.
Paraformaldehyde (Aldrich)	−34.1	n.a.	n.a.	n.a.

n.a.: Not available.
[a] δ‰ BMD derivative = (δ‰ hydrocortisone × 21/23) + (δ‰ paraformaldehyde × 2/23).
[b] Δδ between calculated and measured δ‰ of hydrocortisone BMD derivatives.

+1.5 to +1.6) but this modification is reproducible. Pharmaceutical Hydrocortisone Roussel® gave a BMD derivative with carbon δ‰ in the same range as other synthetic hydrocortisone derivatives (Table 2).

The results of the isotopic measurement of urinary hydrocortisone BMD derivatives are reported in Table 3.

The δ ^{13}C values for natural hydrocortisone from baseline urine are homogeneous (mean −26.7, C.V. 1.7%). Values for hydrocortisone from post-administration urines (mean −31.6 or −31.4, C.V. 3.4 or 3.8% when considering the 30-min or the 2-h post-administration urine from horse C, respectively) are significantly different ($p=0.0019$ or $p=0.003$, respectively). Values for hydrocortisone from post-race urines are homogeneous (mean −26.9, C.V. 1.9%) and not significantly different from the experimental baseline urines. In other words, there is no indication of exogenous contribution from synthetic hydrocortisone.

Table 1 shows that the urinary hydrocortisone concentrations are higher in post-race urines than in experimental baseline urines. This is because they have been increased by exercise [14].

Although carbon isotope ratios from natural and synthetic hydrocortisone were significantly different, it would be of interest to take into account other steroids: precursor(s) and metabolite(s) of hydrocortisone (i.e., pregnanediol and 20β-dihydrocortisol, respectively [15,16]). The isotopic ratio in the precursors should not be affected by exogenous contri-

Table 3
Carbon δ‰ of hydrocortisone BMD derivative from urine samples

Horse	Urine	Type[a]	Hydrocortisone BMD derivative δ‰	Δδ[b]	Δδ%[c]
A	1	Baseline	−26.2		
	4	Treated	−32.8	6.6	25.2
B	2	Baseline	−26.8		
	5	Treated	−30.9	4.1	15.3
C	3	Baseline	−27.1		
	6	Treated	−31.0	3.9	14.4
	7	Treated	−30.6	3.5	12.9
D	8	Unknown	−26.5	—	—
E	9	Unknown	−26.5	—	—
F	10	Unknown	−27.7	—	—
G	11	Unknown	−27.1	—	—
H	12	Unknown	−27.3	—	—
I	13	Unknown	−26.5	—	—

[a] See Table 1.
[b] Δδ = δ‰ baseline − δ‰ treated.
[c] Δδ% = (δ‰ baseline − δ‰ treated)/(δ‰ baseline) × 100.

bution, as observed in testosterone carbon isotopic studies in humans [5]. To interpret data from a horse suspected of having received hydrocortisone, it would be best to take into account all the available data, namely hydrocortisone concentration and carbon isotope ratio compared to precursor and metabolite.

In the context of doping control, efficient doping is achieved when synthetic hydrocortisone from exogenous administration is present in relatively high proportion in horse body fluids. In that case, detection by carbon isotope ratio measurement should be possible.

This method cannot detect the abuse of adrenocorticotrophic hormone (ACTH). ACTH induces hydrocortisone biosynthesis. It is available as a drug and its use in race horses is banned at the time of the race. Upon ACTH administration, the urinary concentration of natural hydrocortisone would increase, but its carbon isotope ratio would reflect that it is natural.

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