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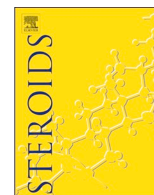

Separation and identification of the epimeric doping agents – Dexamethasone and betamethasone in equine urine and plasma: A reversed phase chiral chromatographic approach

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ABSTRACT

Chirality is one of the most important considerations when controlling doping. The epimeric corticosteroids dexamethasone and betamethasone are significantly potent and long-acting, and they are highly abused in equestrian sports. The scope of this study was to develop a simple and reliable analytical method for simultaneously identifying and separating regularly abused co-eluting corticosteroids in equine urine and plasma. In this paper, we present a simple and rapid method for the chiral separation and identification of epimeric mixtures of dexamethasone and betamethasone using a Thermo Q Exactive high resolution accurate mass spectrometer. The high resolution accurate mass spectrometer system provided extremely high sensitivity, enabling detection of each isomer at a very low concentration from complex biological matrices. Chromatographic separation was performed using amylose and cellulose chiral columns. Reversed phase media showed very good potential for providing a successful chiral resolution in LC-MS analysis. This study also focused on optimizing the mobile phase for elution strength, nature of the organic modifier, additives, and column temperature.

1. Introduction

Synthetic glucocorticoids are often administered to livestock to treat inflammatory diseases. They can also be used to improve feed intake and stimulate growth in livestock. Since the glucocorticoids residues in animal-derived food is suspected to be harmful to humans, the uses of certain substances with hormonal or thyrostatic action as growth-promoting additives have been banned by the European Commission [1] [Table 1]. Dexamethasone [2–4] and betamethasone [5–7] (Fig. 1) are the potent, long-acting epimeric corticosteroid drugs with approximately thirty times more anti-inflammatory and immunosuppressive effects than that of cortisol [8–13]. They are also used to treat recurrent airway obstruction. These drugs are highly abused in the field of equestrian sports.

Chiral substances possess a unique feature that, despite sharing identical molecular formula, and bonding distances, they cannot be superimposed [14]. The significant difference in the physiochemical and biochemical properties of racemic mixtures and individual chiral isomer prompted us to study the chiral separation and detection. In forensic toxicology, chiral analysis have been vital in the correct interpretation of drugs of abuse and other "doping" agents. As the differential actions and toxicities of enantiomers became more evident, the technology for chiral drug development and detection also advanced [15,16]. According to the guidelines, composition of a chiral drug had to be known when applied in pharmacological, toxicological, and clinical studies [17]. One isomer of a chiral drug may have a desired beneficial effect while the other may be inactive, or cause undesirable side effects [18,19]. Hence, in forensic toxicology it has become increasingly important to identify the chirality of the doping agents to avoid legal arguments and challenges to the analytical findings [20–22]. In this context different UHPLC-MS/MS methods with shorter runtime were developed and validated by researchers [23–25]. But the reported separation and identifications were quite tedious and success rate is highly dependent on the performance of the column and other analytical conditions.

To support drug metabolism and pharmacokinetic studies of chiral drugs, it is necessary to combine the resolving power of HPLC with the sensitivity of mass spectrometric techniques. Chiral LC-MS methods must be selective, fast, robust, and sensitive enough, to identify the distomer is at low levels in the presence of the eutomer. They must also be free of interferences from the variety of components present in a complex biological matrix such as blood, tissue, or urine samples.

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Table 1
European Union maximum residue limits for dexamethasone and betamethasone.

Substance	Animal species	MRL (μg/kg)	Target tissues
Dexamethasone	Cattle	0.30	Milk
	Cattle, pig, horse	0.75	Muscle
		2.00	Liver
		0.75	Kidney
Betamethasone	Cattle	0.30	Milk
		0.75	Muscle
		2.00	Liver
		0.75	Kidney
	Pig	0.75	Muscle
		2.00	Liver
		0.75	Kidney

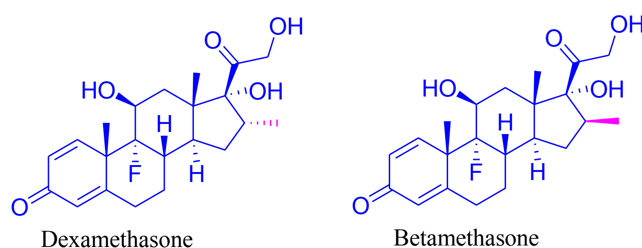

Fig. 1. Chemical structures of the epimeric drug molecules evaluated in this study.

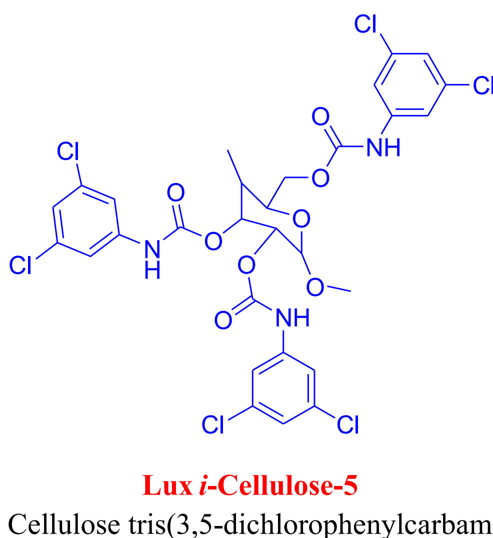

Fig. 2. Chemical structures of the chiral stationary phase evaluated in this study.

Advancements in column technology have improved the efficiency of chiral stationary phases used in routine chiral separations [26–28].

Phenomenex Lux polysaccharide columns offer a wide range of chiral separations of racemic components. Amylose-1 [amylose tris (2, 5-dimethylphenylcarbamate)], amylose-2 [amylose tris (2-chloro-5-methylphenylcarbamate)], cellulose-1 [cellulose tris (3, 4-dimethylphenylcarbamate)] and cellulose-2 [cellulose tris(3-chloro-4-methylphenylcarbamate)] stationary phases provide complex chiral recognition that greatly increases the chances of achieving chiral resolution [29]. Lux i-cellulose-5 (Fig. 2) contain a chemical cross-linking between the polysaccharide and silica supports which in turn provides incredible strong solvent robustness compared to coated phases and prevents the phase from being dissolved in strong solvents.

2. Materials and methods

2.1. Experimental materials

HPLC grade methanol, tetrahydrofuran, ethyl acetate, dichloromethane and methyl *t*-butyl ether were procured from Fisher Chemicals, Germany. LC-MS grade ethanol, n-hexane and acetonitrile were obtained from Merck KGaA, Germany, while formic acid, acetic acid, trifluoroacetic acid, ammonium formate (LC-MS grade), dexamethasone, betamethasone and D_4-hydrocortisone were procured from Merck, USA. Isopropyl alcohol was obtained from Sisco Research Laboratories, Mumbai, India. HF Bond Elut C18, 3CC, 500 mg cartridge was acquired from Agilent Technologies, USA. The columns used for the separation were bought from different suppliers. Lux cellulose-1, Lux cellulose-2, Lux i-cellulose-5, Lux amylose-1, Lux i-amylose-1 and Lux amylose-2 were obtained from Phenomenex, USA, and YMC-amylose-C was obtained from YMC, USA.

2.2. Sample preparation

2.2.1. Urine

The urine sample was subjected to solid phase extraction procedure using Agilent HF Bond Elut C18 cartridge. The samples were adjusted to pH 4.8, β-Glucuronidase from *Escherichia coli* was added (2000 units/mL of the sample) and enzymatic deconjugation was carried out overnight at 37 °C. The pH of the sample was then adjusted to 7.0 using 4.0 N aqueous HCl and 10% aqueous ammonia. Sample was centrifuged (Thermo Fisher Scientific, Heraeus Megafuge 16, 5000 rpm, 15 min) for settling the suspended particles. The supernatant (5.0 mL) was pipetted into a clean test tube and the internal standard (D_4-hydrocortisone; 50 μL; 1.0 μg/mL) was added and vortexed. The C18 cartridge was conditioned with methanol (2.0 mL) followed by water (2.0 mL). The urine sample was passed through the cartridge slowly at a flow rate of 1.0 mL/min under vacuum. The cartridge was then washed with 2.0 mL de-ionized water and dried under vacuum for 2.0 min. Subsequently, the cartridge was eluted with 5.0 mL of dichloromethane and ethyl acetate mixture (4:1) and the eluent was washed with 0.2 M aqueous sodium hydroxide. The organic layer was transferred to a Kimble tube and dried under nitrogen at 60 °C. Further, the sample was reconstituted with 1:1 methanol-water mixture (50 μL), transferred to HPLC auto-sampler vial and then injected (10 μL) into LC-MS system for analysis.

Table 2
LC method optimization for dexamethasone and betamethasone separation.

SI No.	Column	Mobile phase A	Mobile phase B	Method type*	Resolution (R)
1	Lux cellulose-1	0.1% formic acid in water	0.1% formic acid in acetonitrile	Isocratic	0.53
2	Lux cellulose-2	0.1% formic acid in water	0.1% formic acid in acetonitrile	Isocratic	0.80
3	Lux i-cellulose-5	0.1% formic acid in water	0.1% formic acid in acetonitrile	Isocratic	1.11
4	YMC-amylose-C	0.1% formic acid in water	0.1% formic acid in acetonitrile	Isocratic	0.00
5	Lux amylose-1	0.1% formic acid in water	0.1% formic acid in acetonitrile	Isocratic	0.70
6	Lux amylose-2	0.1% formic acid in water	0.1% formic acid in acetonitrile	Isocratic	0.45

* Isocratic; 50% mobile phase A: 50% mobile phase B, run time 10 min.

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