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Comparison of the efficiences of enzymatic and chemical hydrolysis of (nortestosterone and diethylstilboestrol) glucuronides in bovine urine

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Abstract

Residues of 19-nortestosterone (19-NT) and diethylstilboestrol (DES) are excreted in bovine urine, mainly conjugated to glucuronic acid. Prior to quantification, urine must be deconjugated, which is commonly performed by enzymatic or chemical hydrolysis. The efficiencies of two enzymatic and two chemical deconjugation methods were studied. The range of efficiencies obtained for DES were 51.8% (β-glucuronidase, incubation at 37°C overnight) and 2.7% (methanolic HCl), respectively. Similarly, efficiencies for NT ranged from 43.1% (β-glucuronidase, incubation at 55°C for 2 h) to 12.7% (methanolic HCl). The results highlight that within control laboratories significant underestimation of drug residue content in samples may occur, due to poor deconjugation.

Keywords: Nortestosterone; Diethylstilbestrol

1. Introduction

The use of growth promoting drugs for fattening livestock has been banned in the European Union since 1986 [1]. This has resulted in the development of a broad range of methods for detecting residues of these drugs in biological matrices. In this laboratory, enzyme linked immunosorbent assays (ELISAs) have been developed to detect residues of two growth promoting drugs 19-nortestosterone (NT) and diethylstilboestrol (DES) [2]. NT is an anabolic steroid and its illegal use as a growth promoter has been widely reported throughout Europe [3,4]. DES is a synthetic estrogen and was banned as a growth promoter in food producing animals in 1979 by the US Food and Drug Administration (FDA). Due to the relative ease of sample collection and pharmacokinetic considerations, urine is commonly chosen as the target matrix for detecting illegal administration of NT and DES. It has been established that residues of these drugs in bovine urine, are found conjugated mainly to glucuronic acid and to a much lesser extent, sulfuric acid [5–7]. Therefore, prior to extraction and subsequent quantification of the drug residue content, ''deconjugation'' of these acid metabolites to organic soluble compounds is required. This is a very time consuming process because some drugs may be relatively unstable and

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therefore require that deconjugation be carried out under mild conditions. Generally, deconjugation of acidic drug metabolites is carried out either by enzymatic [8,9] or chemical hydrolysis [10]. It was however unclear as to which of these procedures is most suited to a control laboratory environment, where accuracy and speed are often conflicting requirements.

The aim of the current study was to investigate the efficiencies of four deconjugation procedures, two by enzymatic hydrolysis and two using chemical hydrolysis for NT and DES. Bovine urine was spiked with the glucuronide form of each drug and was submitted to each of the deconjugation procedures. The amount of ''free'' drug was subsequently determined by gas chromatography–mass spectrometry (GC–MS).

2. Experimental

2.1. Chemicals and reagents

19-Nortestosterone (4-estren-17β-ol-3-one) (NT), 19-nortestosterone 17-(β-D-glucuronide) (17β-hydroxy-19-nor-4-androsten-3-one 17-glucuronide) (NT-gluc), diethylstilbestrol (stilbestrol) (DES), diethylstilbestrol glucuronide (DES-gluc), acetyl chloride (ACS reagent), trimethylchlorosilane and β-glucuronidase, type H-2 from *Helix pomatia*, with a β-glucuronidase activity of approximately 100 000 units ml^{-1} at pH 5.0 and a sulfatase activity of 5000 units ml^{-1} were obtained from Sigma (Poole, UK). High-performance liquid chromatography (HPLC)-grade methanol and ethanol were obtained from Rathburn (Walkerburn, UK). Diethyl ether (HPLC grade) was obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Acetone (HPLC grade) was obtained from BDH (Poole, UK) and was dried over molecular sieve. Heptafluorobutyric acid anhydride (HFAA) and 3M Emphaze Biosupport Medium were obtained from Pierce and Warriner (Chester, UK). All other chemicals used were supplied by BDH and were of Analar grade. Stock solutions of the steroids (10 μg ml^{-1}) were prepared in methanol and stored at 4°C until used. 0.2 *M* Monobasic sodium phosphate solution was prepared by dissolving sodium dihydrogen orthophosphate 1-hydrate (13.799 g) in 500 ml of deionised water and 0.2 *M* dibasic sodium phosphate solution was prepared by dissolving anhydrous disodium dhydrogen orthophosphate (14.195 g) in 500 ml of deionised water. 0.1 *M* Phosphate buffer (pH 7.4) was prepared by adding 0.2 *M* monobasic sodium phosphate solution (19.0 ml) to 0.2 *M* dibasic sodium phosphate solution (81.0 ml) and diluting to a total volume of 200 ml with deionised water. 0.1 *M* Phosphate buffer (pH 6.5) was prepared by adding 0.2 *M* monobasic sodium phosphate solution (30.0 ml) to 0.2 *M* dibasic sodium phosphate solution (70.0 ml) and diluting to a total volume of 200 ml with deionised water. 1 *M* Anhydrous methanolic hydrogen chloride was prepared by adding acetyl chloride (39.25 g), dropwise, to 500 ml of methanol. 1 *M* trimethylchlorosilane (TMCS) solution was prepared by adding trimethylchlorosilane (54.3 g) to 500 ml of methanol.

2.2. Apparatus

The robotic sample processor used was a RSP 5052 purchased from Tecan UK (Reading, UK). Other instruments used were a vortexer (Fisons Scientific Equipment, Loughborough, UK), a sonicator (Decon Labs., East Sussex, UK), an end-over-end mixer (Luckham, Sussex, UK), an IEC Centra-8R centrifuge (International Equipment Company, MA, USA), a water-bath (Grant, Cambridge, UK), a pH meter (Jenway 3010, Essex, UK), an incubator (Laboratory Thermal Equipment, Oldham, UK) and a DB-3A Dri-block with sample concentrator (Techne, Cambridge, UK). Disposable 3-ml polypropylene columns and porous polyethylene 20 μm frits were obtained from Isolute Accessories (Mid Glamorgan, UK). Sep-Pak C_{18} (500 mg) cartridges were obtained from Waters (Milford, USA). Tapered glass vials, 500 μl, with caps were obtained from Chromacol (Herts., UK). The gas chromatography (GC) system used was a HP 5890 Series II interfaced with an HP 5972A mass spectrometer obtained from Hewlett-Packard (Palo Alto, CA, USA).

2.3. Preparation of immunoaffinity chromatography (IAC) columns

Polyclonal antibodies to DES and 19-NT were raised in a rabbit and goat, respectively, by the

immunization procedure of McCaughey et al. [11]. The specificity of the antibodies were assessed by determining the extent to which each cross-reacted with compounds of a similar chemical structure to that used to raise the antibody. The antibodies were purified by precipitation with 50% ammonium sulfate by the procedure of Harlow and Lane [12] prior to coupling to 3M Emphase Biosupport Medium by the procedure described by Fodey et al. [2].

2.4. Source of samples

Urine was collected ante mortem from animals born into an experimental unit and known to have been reared under nortestosterone- and diethylstilbestrol-free conditions. These fluids were pooled and stored at −20°C until required.

2.5. Sample preparation

2.5.1. Nortestosterone samples

Pooled urine (6 ml) was placed into a glass universal, in duplicate and 10 μg ml^{-1} nortestosterone stock solution (30 μl) was added, to produce samples containing 50 ng ml^{-1} NT. Similarly, nortestosterone glucuronide stock solution (30 μl) was added to pooled urine (6 ml) in duplicate. 0.2 *M* sodium acetate solution, pH 5.0 (3 ml) and 17β-D_3-19-nortestosterone (30 μl of 1 μg ml^{-1} in ethanol) internal standard were added to all the samples.

2.5.2. Diethylstilbestrol samples

Urine samples were prepared in duplicate, as before, though using 10 μg ml^{-1} diethylstilbestrol and 10 μg ml^{-1} diethylstilbestrol glucuronide stock solutions, to produce samples containing 50 ng ml^{-1} DES. 0.2 *M* sodium acetate solution, pH 5.0 (3 ml) and D_6-DES (100 μl of 100 ng ml^{-1} in ethanol) internal standard was added to all of these samples.

All the prepared samples (nortestosterone and diethylstilboestrol) were subjected to each of four deconjugation methods. For both drugs, blank samples were prepared using the conjugated forms of the two drugs. The experiment was repeated three times for 19-NT steroid and twice more using DES giving a total of 96 and 72 samples, respectively.

2.6. Method 1 (enzyme, incubation at 37°C)

β-Glucuronidase (120 μl) was added to each of the samples, except the blanks. All the samples were adjusted to pH 5.0 using 1 *M* acetic acid, capped and incubated overnight, in a waterbath, at 37°C. The cooled samples were adjusted to pH 10.0 with 1 *M* sodium hydroxide. Diethyl ether (5 ml) was added, the samples were gently shaken on an end-over-end mixer for 1 min and centrifuged at 2700 *g* for 10 min. The upper ether layer was removed and collected, in glass tubes, using a pasteur pipette. The extraction was repeated and the ether layers combined. The ether was evaporated to dryness, under nitrogen, using a Dri-block and sample concentrator at 50°C.

2.7. Method 2 (enzyme, incubation at 55°C)

As method 1 except that all the samples were incubated, for 2 h, in a waterbath, at 55°C.

2.8. Method 3 (methanolysis by methanolic HCl)

Sep-Pak C_{18} (500 mg) cartridges were conditioned, prior to use, by the sequential addition of methanol (3 ml) and deionised water (3 ml). The samples (6 ml) were applied to the cartridges and allowed to run through under gravity. The cartridges were washed with deionised water (3 ml) and the samples eluted with methanol (4 ml) collecting the eluents in glass universals. The solvent was evaporated to dryness, under nitrogen, using a Dri-block and sample concentrator at 60°C. Methanolic hydrogen chloride (0.5 ml) was added to each of the samples, except the blanks. All the samples were transferred to an incubator, for 10 min, at 60°C. The solvent was evaporated to dryness, under nitrogen, using a Dri-block and sample concentrator at 60°C. The cooled samples were resuspended in three drops of methanol and 0.1 *M* phosphate buffer, pH 6.5 (0.5 ml). Diethyl ether (2.5 ml) was added, the samples were gently shaken on an end-over-end mixer for 1 min and centrifuged at 2700 *g* for 10 min. The upper ether layer was removed and collected, in glass tubes, using a pasteur pipette. The extraction was repeated and the ether layers combined. The ether was evaporated to dryness, under nitrogen, using a Dri-block and sample concentrator at 50°C.

2.9. Method 4 (methanolysis by methanolic TMCS)

As method 3, except, after elution of samples from C_{18} columns,1 *M* TMCS solution (1.0 ml) was added to each of the samples, except the blanks. All the samples were incubated for 1 h, in a waterbath, at 55°C.

2.10. Immunoaffinity clean-up

The dry samples, from each of the four methods, were resuspended in ethanol (200 μl), vortexed for 30 s, sonicated for 10 min and phosphate buffer, pH 7.4 (5.2 ml) added. The samples were then applied to prepared IAC columns, washed and eluted with 80% ethanol (3.2 ml), using a Robotic Sample Processor (RSP). The eluents were evaporated to dryness, under nitrogen, using a Dri-block and sample concentrator at 85°C. The cooled tubes were resuspended in methanol (500 μl), capped, vortex-mixed for 30 s and sonicated for 10 min. The methanol solution was transferred to tapered glass sample vials and subjected to analysis by GC–MS.

2.11. GC–MS analysis

Internal recovery standards for 19-NT were prepared by adding 10 μg ml^{-1} of NT stock solution (25 μl), 10 μg ml^{-1} of NT-glucuronide stock solution (25 μl) and 1 μg ml^{-1} of 17β-D_3-19NT (25 μl) together, into tapered glass vials. Similarly, internal recovery standards for DES were prepared by adding 10 μg ml^{-1} of DES stock standard (25 μl), 10 μg ml^{-1} of DES-glucuronide stock standard (25 μl) and 100 ng ml^{-1} of D_6-DES (100 μl) into tapered glass vials. All sample extracts and standards were dried down, under nitrogen, at 50°C. Dry acetone (200 μl) was added to all vials and vortex-mixed. HFAA (50 μl) was added and the vials vortex-mixed again. The vials were capped, placed in a Dri-Block, at 60°C, and allowed to derivatize for 1 h. During derivatization the vials were vortex-mixed every 20 min. The derivatized extracts were dried down under nitrogen, at 60°C, redissolved in toluene (100 μl), vortexed and sonicated for 10 min. The toluene extracts were transferred to tapered vials and capped. The samples were then analysed on the GC–MS system in electron ionization mode.

Identification of 19-NT was by single ion monitoring (SIM) of the HFAA derivative at *m*/*z* 666. Quantification was carried out by calculating the ratio of the molecular ion (*m*/*z* 666) to that of the D_3 internal standard (*m*/*z* 669) in the sample and comparing this with the ratio of the standard. The method was linear over the range 0–40 ng ml^{-1} and the limit of detection was 0.2 ng ml^{-1}. The relative standard deviation (RSD) of the method was 5.0% ($n=15$). Using a negative urine spiked at 5 ng ml^{-1}, the mean recovery was 96% ($n=15$).

Identification of DES was by SIM of the HFAA derivative at *m*/*z* 660. Quantification was carried out by calculating the ratio of the molecular ion (*m*/*z* 660) to that of the D_6 internal standard (*m*/*z* 666) in the sample and comparing this with the ratio of the standard. The method was linear over the range 0–40 ng ml^{-1} and the limit of detection was 0.1 ng ml^{-1}. The relative standard deviation (RSD) of the method was 12.7% ($n=18$). Using a negative urine spiked at 1 ng ml^{-1}, the mean recovery was 110% ($n=18$).

3. Results

3.1. Antibody cross-reactivity profiles

The cross-reactivities of the 19-NT and DES antibodies used in the IAC clean-up procedure are given in Table 1. The 19-NT antibody cross-reacted 100% with β-NT, 22.9% with α-NT and 141% with 4-estren-3,7 dione. The DES antibody cross-reacted 100% with hexoestrol, 34.7% with DES and 4.57% with dienoestrol.

3.2. Comparison of deconjugation methods

The yields of ''free'' drug following deconjugation, by each of the four methods, of 19-NT spiked urine have been summarised in Table 2. The efficiencies of the four methods expressed as percentages are given in Table 3. Method 2 (enzyme, incubation at 55°C) produced the greatest efficiency for deconjugating nortestosterone glucuronide, with a mean yield of 43.1%. Methods 1 and 4 gave comparable results, with means of 34.8 and 33.4%, respectively. Method 3 gave the lowest efficiency at only 12.7%.

The yields of ''free'' drug following deconjuga-

Table 1
Percentage cross-reactivities of associated analytes with antibodies used on IAC columns

Cross-reacting analytes	19-NT antibody (%)	DES antibody (%)
17β-19-Nortestosterone (5-estren-17β-ol-3-one)	100	
17α-19-Nortestosterone (5-estren-17α-ol-3-one)	22.90	
Testosterone (4-androsten-17β-ol-3-one)	1.20	<0.01
Epitestosterone (4-androsten-17α-ol-3-one)	<0.01	
19-Norandrosten dione (4-estren-3,7 dione)	141.00	
5β-Estran-3β-ol-17-one	0.37	
5α-Estran-3α,17β-diol	0.34	
5α-Estran-3β,17α-diol	0.33	
Progesterone	3.20	<0.01
17α-Oestradiol [1,3,5(10) Estratrien-3,17α-diol]	<0.01	
17β-Oestradiol [1,3,5(10) Estratrien-3,17β-diol]	<0.01	<0.01
Diethylstilbestrol		34.7
Hexoestrol		100
Dienoestrol		4.57

Table 2
The yields of free nortestosterone, from NT and NT-glucuronide spiked urine samples, following deconjugation by four different methods

Method	Assay 1		Assay 2		Assay 3		Assay 4		Mean	
	NT (ng ml^{-1})	NT-gluc (ng ml^{-1})	NT (ng ml^{-1})	NT-gluc (ng ml^{-1})	NT (ng ml^{-1})	NT-gluc (ng ml^{-1})	NT (ng ml^{-1})	NT-gluc (ng ml^{-1})	NT (ng ml^{-1})	NT-gluc (ng ml^{-1})
1	70.9	22.0	60.1	19.0	56.8	19.9	42.7	17.8	57.6±11.6	19.7±1.8
2	30.4	15.0	54.0	21.6	61.8	22.8	41.4	19.0	46.9±13.8	19.6±3.5
3	64.4	7.8	56.6	8.6	47.4	8.4	43.9	2.5	53.1±9.3	6.8±2.9
4	61.9	19.5	57.5	16.5	45.1	14.0	41.2	17.5	51.4±9.9	16.9±2.3

Results shown are the calculated mean values of duplicate samples.

tion, by each of the four methods, of DES spiked urine have been summarised in Table 4. The efficiencies of the four methods expressed as percentages are given in Table 5. Method 1 (enzyme, incubation at 37°C) produced the greatest efficiency for deconjugating diethylstilboestrol glucuronide, of 51.8%, closely followed by method 2 with 50.3%. Methods 3 and 4 produced very poor efficiencies of 2.7 and 3.5%, respectively. For all methods the value of the blank samples, which did not undergo deconjugation, were zero (results not shown).

4. Conclusions

In the current study a difference in the efficiencies of hydrolysis was observed between enzymatic deconjugation and methanolysis. Enzymatic hydrolysis

Table 3
The efficiency of four different methods to deconjugate nortestosterone, expressed as percentages

Method	Assay 1	Assay 2	Assay 3	Assay 4	Range	Mean
1	31.0	31.6	35.0	41.7	31.0–41.7	34.8±4.9
2	49.3	40.0	36.9	46.2	36.9–49.3	43.1±5.7
3	12.1	15.2	17.7	5.7	5.7–17.7	12.7±5.2
4	31.5	28.7	31.0	42.5	28.7–42.5	33.4±6.2

Values calculated using expression (NT-gluc/NT)·100.

Table 4
The yields of free diethylstilboestrol, from DES and DES-glucuronide spiked urine samples, following deconjugation by four different methods

Method	Assay 1		Assay 2		Assay 3		Mean	
	DES (ng ml^{-1})	DES-gluc (ng ml^{-1})	DES (ng ml^{-1})	DES-gluc (ng ml^{-1})	DES (ng ml^{-1})	DES-gluc (ng ml^{-1})	DES (ng ml^{-1})	DES-gluc (ng ml^{-1})
1	55.3	29.5	46.1	24.0	39.0	19.5	46.8±8.2	24.3±5.0
2	49.2	28.4	43.8	21.8	43.9	19.0	45.6±3.1	23.1±4.8
3	56.9	1.6	43.1	1.5	36.3	0.6	45.4±10.4	1.2±0.6
4	55.8	2.0	41.7	1.4	32.6	1.1	43.4±11.7	1.5±0.5

Results shown are the calculated mean values of duplicate samples.

gave higher efficiencies than methanolysis for both NT glucuronide and DES glucuronide. One exception was however, for NT glucuronide, when essentially no difference was observed between methanolysis using TMCS and enzymatic deconjugation with an overnight incubation at 37°C. Furthermore, for NT glucuronide, enzymatic deconjugation for 2 h at 55°C gave the highest efficiency of 43.1%. Methanolysis using methanolic hydrogen chloride gave the lowest efficiency of 12.7%. With DES glucuronide, the difference in efficiency between enzymatic deconjugation and methanolysis was even more marked. Both the enzymatic deconjugation methods produced similar efficiencies of around 51%, whereas the two methanolysis methods only gave efficiencies of approximately 3%.

On comparing the two chemical hydrolysis methods it was observed that only methanolysis using TMCS for NT glucuronide produced substantive deconjugation. Acid-catalysed methanolysis was first described by Tang and Crone [13] who used a mixture of 1 *M* acetyl chloride in methanol, consequently producing anhydrous hydrochloric acid by a strong exothermic reaction, for the simultaneous cleavage of steroid glucuronides and sulfate conjugates in equine urine. This procedure has the shortest reaction time of the four methods discussed, of only 10 min. Tang and Crone reported efficiencies of 95 and 85% from the methanolysis of testosterone sulfate and testosterone glucuronide, respectively. Dehennin et al. [14] observed a similar property for TMCS in methanol. They proposed that in a mixture of 1 *M* TMCS in methanol, sufficient hydrochloric acid is released to produce enough of the active intermediate $[CH_3CH_2]^+Cl^-$, which is the cleaving agent of both glucuronic ethers and sulfate esters. A further advantage is that on mixing with methanol, no special care was required. This methanolysis step was a key element of an analytical screening procedure, developed to evaluate androgen conjugates in the post-competition urine of sportsmen. A disadvantage of methanolysis over enzymatic hydrolysis is that drug conjugates must initially be extracted from the urine before hydrolysis can be performed. In addition Orlov et al. [10] reported that acid hydrolysis in the human urine matrix was undesirable because substances formed by the action of the acid on organic urine components, can contaminate the solution. In the present study, the extraction of the drug conjugates was performed by solid-phase extraction (SPE) using Sep-Pak C_{18} cartridges. This step may possibly reduce recoveries of some drug glucuronides, but most authors report recoveries of over 90% for steroid glucuronides [15]. From the

Table 5
The efficiency of four different methods to deconjugate diethylstilboestrol expressed as percentages

Method	Assay 1	Assay 2	Assay 3	Range	Mean
1	53.3	52.1	50.0	50.0–53.3	51.8±1.7
2	57.7	49.8	43.3	43.3–57.7	50.3±7.2
3	2.8	3.5	1.7	1.7–3.5	2.7±0.9
4	3.6	3.4	3.4	3.4–3.6	3.5±0.1

Values calculated using expression (DES-gluc/DES)·100.

present study it was concluded that methanolysis was inadequate in hydrolysing glucuronides of some growth promoting drugs.

On comparing the two enzymatic deconjugation methods it was observed that, in general, incubating for 2 h at 55°C, gave the highest degree of hydrolysis efficiency. Adoption of this method, in a control laboratory, would produce considerable savings on time without any loss in hydrolysis efficiency. It has been widely reported that using *Helix pomatia* in deconjugating steroid conjugates, can produce chemical transformations ''artefacts'' of some steroids [16,17]. For example, if androst-5-ene-3β,17β-diol or pregnenolone are present in the sample, incubation with *Helix pomatia* could give rise to testosterone and progesterone, respectively, being formed. Artefact formation has resulted in some researchers choosing β-glucuronidase from *Escherichia coli* to hydrolyse steroid conjugates [14]. However β-glucuronidase from *E. coli* possesses low enzyme activity for glucuronides and a high activity for sulfates. This enzyme is therefore unsuitable for hydrolysing growth promoting drugs in bovine urine. Vanluchene et al. [16], further reported that the degree of steroid conversion during incubation with *Helix pomatia* could be substantially reduced by decreasing the incubation time.

In the present study, it was found that both enzymatic deconjugation and methanolysis were unable to completely hydrolyse NT and DES glucuronides. This is in agreement with previous studies. Le Bizec et al. [18] reported incomplete hydrolysis of anabolic steroids using *Helix pomatia*, even under optimum conditions, with chemical hydrolysis being required to produce 100% free steroid. Orlov et al. [10] used acid hydrolysis combined with solvent extraction, which required refluxing urine with hydrochloric acid for 20 min at 80°C. This extreme step is however, unsuitable for laboratories were high sample throughput is required.

In the present study, the highest hydrolysis efficiency obtained was a mean value of 51.8% for DES glucuronide using enzymatic deconjugation with an overnight incubation at 37°C. From the current study it can be concluded that the deconjugation method for each drug conjugate requires optimisation. It is also important to note that if, as in the case of the present study, deconjugation efficiencies are only in the region of 50%, then significant underestimation of drug content in samples may be reported. In addition, generally the efficiency of deconjugation is not used as a parameter in validating analytical methods, therefore, it can be seen that detection limits claimed may be significantly higher than desired. The present results underline the requirement to develop alternative, more effective, procedures.

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