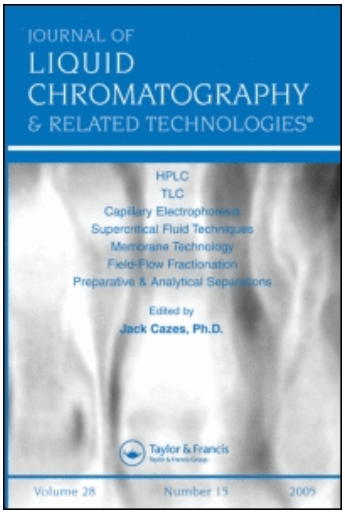

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Multi-Dimensional Liquid Chromatography Method for Determination of Androgen Hormone Residues in Cattle Liver

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To cite this Article Stubbings, George W. and Shepherd, Martin J.(1993) 'A Multi-Dimensional Liquid Chromatography Method for Determination of Androgen Hormone Residues in Cattle Liver', Journal of Liquid Chromatography & Related Technologies, 16: 1, 241 — 255
To link to this Article: DOI: 10.1080/10826079308020909
URL: http://dx.doi.org/10.1080/10826079308020909

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(1), 241-255 (1993)

A MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY METHOD FOR DETERMINATION OF ANDROGEN HORMONE RESIDUES IN CATTLE LIVER

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ABSTRACT

A multidimensional HPLC method is described for the determination of trenbolone and 19-*nor*testosterone steroid hormone residues in cattle liver. Sample extracts are defatted by size exclusion chromatography. The evaporated residue is injected onto a high performance gel permeation column and the two fractions containing the steroids are diverted onto individual silica concentration columns, where the hormone residues are retained. The trapped hormones are then determined separately. Trenbolone held on one concentration column is eluted onto a cyano HPLC column and the steroid peak from this is transferred onto a final analytical silica HPLC column. After re-equilibration of the system the second concentration column is treated similarly for determination of 19-*nor*testosterone. Detection is by UV at 340 nm for trenbolone and 247 nm for 19-*nor*testosterone. Detection limits in cattle liver are 0.1 μg/kg for trenbolone and 0.3 μg/kg for 19-*nor*testosterone. For liver spiked at 2 μg/kg, recoveries of 59% and 39%, with CVs of 7% and 10% were obtained for trenbolone and 19-*nor*testosterone respectively. The low overall recovery for 19-*nor*testosterone was primarily attributable to losses during extraction.

INTRODUCTION

Steroid hormones can be used as weight gain improvers in livestock but are not permitted for use in the European Community in animals intended for human consumption. When employed for this purpose, any residues in edible tissues are likely to be present at levels considerably less than 10 μg/kg. Thus there exists a need for effective methods of analysis of these compounds in tissue at the lowest possible concentrations.

Androgen and gestagen steroidal hormones generally lack structural features which would enable their facile isolation from tissue extracts, unlike the estrogens which may be purified by virtue of their phenolic properties. This, together with the low residue levels of interest, causes problems for the analyst. Current methods [1] depend heavily upon selectivity of detection, either through mass spectrometry or immunoassay. With both of these techniques extensive sample preparation is still required to produce extracts clean enough to permit reliable confirmation of the presence of steroid hormones at residue levels. This is despite employing capillary GC or HPLC separations before the determination step.

Effective analytical separations require a balance of chromatographic selectivity and efficiency. Because selectivity for steroids is low, column efficiency becomes more important. Column switching techniques offer an attractive route for increasing the resolving power of chromatographic systems [2]. In practice, this is the on-line and higher performance analogue of conventional off-line cleanup which typically employs a sequence of low-resolution chromatographic steps. LC-LC switching systems are readily assembled, although some care is necessary in selection of compatible chromatographic modes to ensure refocussing of analyte peaks transferred between columns. Combinations of LC and GC permit substantially higher overall efficiency but are more restrictive because of the natures of the two mobile phases and the volatility constraints inherent in GC.

On-line LC-GC column switching has been applied by Grob *et al.* [3] to the determination of diethylstilbestrol (DES) in cattle urine; hydrolysed urine was passed through a solid phase extraction cartridge; the eluted residue was derivatised with pentafluorobenzyl bromide and then separated by normal phase HPLC and capillary GC. A detection limit of *ca* 0.2 μg/kg was claimed when using electron capture detection.

LC-LC switching has been developed by Henion and colleagues who applied coupled normal phase HPLC on phenyl and silica columns, with UV detection, to the determination in cattle kidney, liver and muscle of melengestrol acetate [4] (validated at 10 μg/kg, with a detection limit of 3 μg/kg for liver; monitored at 287 nm) and dexamethasone [5] (validated at 30 μg/kg, with a detection limit 6 μg/kg in liver; monitored at 239 nm).

Coupled column systems yield the highest overall efficiency when the underlying mechanisms of separation of the individual columns are as different as possible. Porous poly(styrene-divinylbenzene) columns may be operated under either size exclusion (SEC) or gel permeation (GPC) conditions, depending upon the match between the polarity of the gel and the mobile phase [6]. SEC separations take place where there is no interaction between gel and analyte, and rely upon size differences between solutes. GPC mobile phases superimpose moderate adsorption and partition processes on the fundamental size exclusion mechanism. The major disadvantage of SEC and GPC is an inherently low resolution. Unlike other forms of liquid chromatography, SEC is based essentially upon entropic differences between solutes in the LC system [6]. Retention times are consistent, columns accept high loadings and have lifetimes of thousands of samples. Thus SEC and GPC are ideal candidates for the initial stage in multidimensional systems [7]. This report describes the application of coupled column GPC and normal phase HPLC to the determination of the androgenic steroids trenbolone and 19-*nor*testosterone in animal tissues intended for human consumption.

MATERIALS

17β-19-*Nor*testosterone and β-glucuronidase, types H-1 and H-5, were purchased from Sigma (Poole, UK). 17β-19-[^{3}H]*Nor*testosterone with a specific activity of 1.37 TBq/mmol was from Amersham International (Amersham, UK). 17α-Trenbolone was a gift from Roussel-UCLAF (Paris, France). Water (HPLC grade) was from Fisons (Loughborough, UK); glass distilled grade hexane and toluene and HPLC grade acetonitrile, dichloromethane, methanol, propan-2-ol and 2,2,4-trimethylpentane were from Rathburn (Walkerburn, Scotland). Sodium acetate trihydrate, Analar grade, was from BDH (Dagenham, UK). Bio-Beads S-X3 was from Bio-Rad (Richmond, CA). "Ready Value" scintillation cocktail (Beckman, Fullerton, CA) was employed. Samples of cattle liver were obtained from local retail outlets.

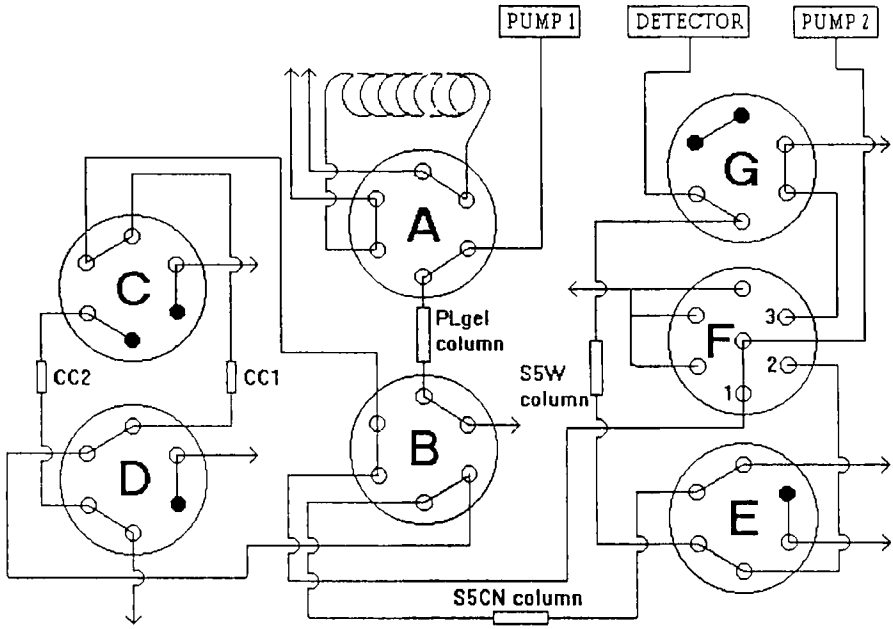

FIGURE 1. Schematic diagram of coupled column analytical system.

For full details, see *Methods*. Ports shown black are blocked off. All arrowheads indicate flow to waste. CC1 and CC2: concentration columns. The flow paths through all valves are shown in position 1. For two-way valves B-E and G, the alternative setting is position 2. For the six-way solvent distribution valve F, positions 1-3 are marked. The injection valve is shown in the load position.

METHODS

Reagents

Acetate buffer was prepared by adjusting aqueous sodium acetate (0.04 M) to pH 4.1 with glacial acetic acid. β-Glucuronidase solution was made up by dissolving the solid (H-1, 230 mg or H-5, 170 mg) in 10 mL acetate buffer. 17β-19-[^{3}H]*Nor*testosterone was purified before use by coupled column normal phase HPLC chromatography using the system described below (see *Coupled Column Chromatography*) after bypassing the PLgel column and loading it directly onto a

concentration column (CC1, Figure 1) in toluene : cyclohexane. The fraction containing labelled hormone was collected after elution from the S5W silica column, evaporated to dryness and redissolved in methanol.

β-Counting

Samples containing 19-[^{3}H]*nor*testosterone were prepared for counting by evaporating aliquots (0.4 mL) to dryness in minivials and mixing with scintillation cocktail (4 mL). Vials were held in darkness to minimise chemiluminescence and β-activity measured on a 1216 Rackbeta II scintillation counter (Pharmacia LKB, Uppsala, Sweden), using external quench correction.

Sample hydrolysis, extraction and three phase partition

Liver (10.0 g) was sliced and placed in a centrifuge bottle. The sample was homogenised with an Ultra-Turrax model T25, (Janke and Kunkel, Staufen, Germany) for 60 s in acetate buffer (20 mL) and the shaft of the homogeniser washed with an additional 2 mL buffer. β-Glucuronidase solution (200 μL) was added, the mixture swirled and then incubated at 37 °C for 16 h. Acetonitrile (40 mL) was added, the sample homogenised for 30 s and then centrifuged. In some experiments with 19-[^{3}H]*nor*testosterone, the pellet was re-extracted into methanol (50 mL) and again centrifuged; the methanolic layer was examined separately for radioactivity. The initial acetonitrile extract was decanted into a 100 mL stoppered measuring cylinder, hexane : dichloromethane (4 + 1, 20 mL) added and the three layers mixed by shaking for 30 s. The layers were allowed to settle for 30-60 min and the middle phase removed by aspiration after recording its volume. An aliquot equivalent to 7.5 g of tissue was mixed with propan-2-ol (20 mL) and evaporated to dryness. The residue was transferred to a 25 mL pear-shaped flask with dichloromethane (3 x 3.0 mL), filtered and evaporated to give a total of about 0.15 mL of an oil which was redissolved in toluene : dichloromethane (1 + 1, 0.2 mL) and transferred to a 2 mL vial, precalibrated to indicate a volume of 0.75 mL. The pear shaped flask was rinsed with aliquots of toluene : dichloromethane (3 x 0.1 mL) which were combined with the initial solution. The total volume was adjusted to 0.75 mL with additional toluene : dichloromethane, and the extract purified by SEC.

Size exclusion chromatography

The system consisted of Gilson (Villiers-le-Bel, France) model 307 pump, 401 dilutor and 232 sample injector / fraction collector. A glass SR 25/45 column (Pharmacia, Uppsala) was packed with Bio-Beads S-X3 (345 x 25 mm) in the mobile phase of toluene : dichloromethane (1 + 1). Elution was at 5 mL/min. An aliquot of the extract (0.50 mL) was injected and the fraction (97.5-137.5 mL) which contained trenbolone and 19-*nor*testosterone was collected. The solvent was evaporated under nitrogen and the residue redissolved in toluene : cyclohexane (1 + 1, 0.625 mL) for coupled column HPLC analysis.

Coupled column chromatography

The system and its initial configuration are shown diagramatically in Figure 1. It consisted of a 7125 sample injection valve (A) fitted with a 1.00 mL loop, five 7010 six-port two-way valves (B-E and G) and a 7060 solvent distribution valve (F) (Rheodyne, Cotati, CA), two 6000A pumps and a 484 UV detector (Waters, Milford, MA). Peak areas were measured with an SP4290 integrator (Spectra Physics). Valves D and E were actuated by Universal Valve Switching Modules (Gilson) controlled by timed events from a Model 222 sample changing unit (Gilson). The S5CN column was thermostatted at 30 °C using a column heater (Jones, Hengoed, UK).

The columns employed for separation were: gel permeation - 100 Å PLgel, 5 µm, 300 x 7.5 mm (Polymer Laboratories, Church Stretton, UK); normal phase - Spherisorb S5CN, (cyano), 5 µm, 250 x 4.6 mm, and Spherisorb S5W, (silica), 5 µm, 250 x 4.6 mm (Hichrom, Reading, UK). Concentration columns 1 and 2 (CC1 and CC2) were ChromSpher silica, 40 µm, 10 x 2.1 mm, (P/N 28671, Chrompack, Middelburg, Netherlands). The mobile phase for the PLgel column was toluene : cyclohexane (1 + 1) at 1.0 mL/min. Elution of hormones from the concentration columns and all normal phase stages of the separations were carried out under isocratic normal phase HPLC conditions using 2,2,4-trimethylpentane : propan-2-ol (85 + 15), at 1.0 mL/min. The detector was set at 340 nm for trenbolone and 247 nm for 19-*nor*testosterone.

An aliquot (0.50 mL, 4.0 gram equivalents of tissue) of the dissolved residue from size exclusion chromatography was injected onto a PLgel gel permeation column. The two fractions containing TB and NT from the PLgel column were passed through separate concentration columns where the hormones were individually retained. Final

analysis of the hormones was then carried out sequentially. TB was eluted from its concentration column and determined by a normal phase column-switching procedure. The analytical columns were then flushed to remove long-retained sample components and NT determined in the same manner. The valve switching events are described in Tables 1 and 2.

RESULTS

Sensitivity of detection was demonstrated by spiking the equivalent of 1.0 μg/kg each of 19-*nor*testosterone and trenbolone into extracts from blank cattle liver immediately before analysis on the coupled column system. Typical chromatograms are presented in Figure 2. Detection limits (signal to noise ratio of 3:1) were found to be 0.1 μg/kg for trenbolone and 0.3 μg/kg for 19-*nor*testosterone. The method was validated by analysis of two batches of 6 cattle liver samples spiked before digestion with 2 μg/kg of each hormone. The individual recoveries, together with means and standard deviations, are shown in Table 3.

Recovery of 19-*nor*testosterone during the extraction and subsequent manipulations was monitored by radiolabel experiments. A total of 0.05 μg/kg of cold and tritiated 19-*nor*testosterone was added, either before or after deconjugation. The distribution of activity between the three phases during the partition step is shown in Table 4. For two of these samples the tissue pellet was re-extracted with methanol, which solubilised an additional 18% of radioactivity (Table 4).

DISCUSSION

One major advantage anticipated for the coupled column method is its applicability to a wide range of different steroidal hormones. Analysis for residues of these compounds in animal muscle and liver is currently carried out in this Laboratory by immunoaffinity chromatography (IAC) cleanup and reverse phase HPLC with UV detection. Although this procedure gives good results it has a number of drawbacks. The very specificity of antibody-antigen recognition means that other antibodies and IAC columns are required if the method is to be extended to a wider range of hormones, such as may be encountered when these drugs are used illegally for meat production. Furthermore, the present generation of IAC columns based on soft gels have low flow rates and thus give rise to extended analysis times, while the gels are

TABLE 1

Sequence of Events for Column Switching Analysis.

STEP	TIME (min)	EVENTS
I	0	Inject sample using valve A and start GPC separation on PLgel column. Backflush of CC1 and S5CN and S5W columns continues from end of previous cycle.
II	10.0	End of backflush. Reset all valves to positions shown in Figure 1. Restabilise system after change of mobile phase flow direction.
III	11.7	Flow from PLgel column diverted through CC1 for 2.0 min; NT trapped.
IV	13.7	Flow from PLgel column diverted through CC2 for 2.5 min; TB trapped.
V	16.2	Isocratic normal phase HPLC started. NT eluted from CC1 onto S5CN column. S5W column off-line.
VI	23.7	Start transfer of NT peak from S5CN column onto S5W column.
VII	24.3	Finish transfer of NT fraction from S5CN column onto S5W column. Total transfer time 0.6 min. S5CN column switched off-line from S5W column. NT determination completed on S5W column; hormone detected at ca 35.5 min.
VIII	39.3	CC1; S5CN and S5W columns backflushed with mobile phase for 15 min.
IX	54.3	Restabilise system after change of mobile phase flow direction.
X	55.3	TB eluted from CC2 onto S5CN column. S5W column off-line.
XI	64.1	Start transfer of TB fraction from S5CN column onto S5W column.
XII	64.7	Finish transfer of TB fraction from S5CN column onto S5W column. Total transfer time 0.6 min. S5CN column switched off-line from S5W column. TB analysis completed on S5W column; hormone detected at ca 75.0 min.
XIII	78.0	CC1, S5CN and S5W columns backflushed for total of 15 min which continues in Step I at beginning of next cycle.
I	83.0	Start of next cycle.

TABLE 2

Valve Position Changes at Each Step in the Column Switching Analysis.

STEP	TIME (min)	Valve B	Valve C	Valve D	Valve E	Valve F	Valve G
I	0	1	2	1	2	3	2
II	10.7	-	1	-	1	1	1
III	11.7	2	-	2	-	-	-
IV	13.7	-	2	1	-	-	-
V	16.2	1	1	-	-	-	-
VI	23.7	-	-	-	2	-	-
VII	24.3	-	-	-	1	2	-
VIII	39.3	-	2	-	2	3	2
IX	54.3	-	1	-	1	1	1
X	55.3	-	2	2	-	-	-
XI	64.1	-	-	-	2	-	-
XII	64.7	-	-	-	1	2	-
XIII	78.0	-	-	1	2	3	2

Note: Position 1 for each of the two-way valves B-E and G is arbitrarily defined as shown in Figure 1. The alternative setting is referred to as position 2. Positions 1, 2 and 3 for distribution valve F are indicated in Figure 1.

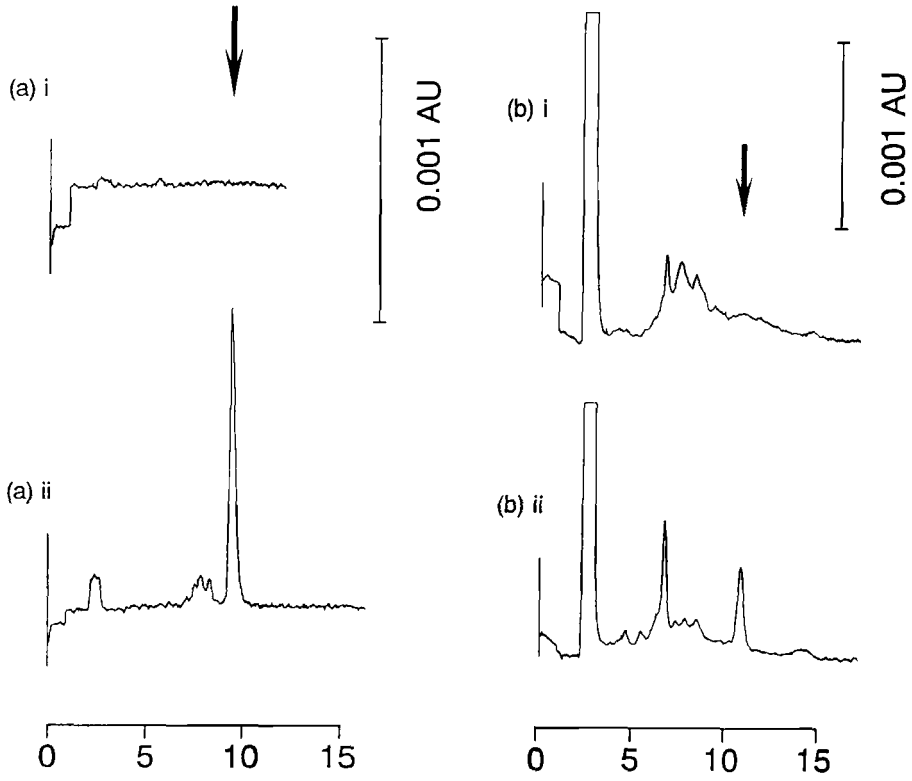

FIGURE 2. Trenbolone and 19-*nor*testosterone spiked at 1 µg/kg into blank cattle liver extracts.

(a) Trenbolone: (i) Blank, (ii) Spike. Horizontal axis: minutes.

(b) 19-*Nor*testosterone: (i) Blank, (ii) Spike.

prone to non-specific adsorption of tissue co-extractives which may present interference problems. IAC columns employing silica or other rigid matrices offer improved flow characteristics but problems with non-specific adsorption might be exacerbated. Another useful feature of the coupled column method is its potential for automation. The current apparatus is only partially automated, but all that is required to complete the system is an autosampler and four additional valve switching modules. The initial SEC defatting stage is completely automated.

TABLE 3

Recoveries of Trenbolone and 19-*Nor*testosterone Spiked at 2 μg/kg into Cattle Liver Prior to *H Pomatia* Treatment.

	Batch 1		Batch 2	
	TB	NT	TB	NT
Sample 1	54.0	32.8	53.9	35.4
Sample 2	56.5	33.3	62.5	45.1
Sample 3	57.9	41.1	-	39.4
Sample 4	58.8	40.4	54.5	37.0
Sample 5	59.2	43.8	66.1	44.0
Sample 6	54.2	37.8	67.8	39.4
Mean	56.8	38.2	61.0	40.1
CV	3.6	10.6	9.5	8.7

TABLE 4

Distribution of Activity of 17β-19-[^{3}H]*Nor*testosterone Spike During Extraction and Three Phase Partition

Sample	Activity in fraction (%)				
	Upper phase	Middle phase	Lower phase	Pellet	Total
Pre-hydrolysis spike					
1	5.8	57.8	7.5	nd	71.1*
2	5.4	64.6	7.6	19.6	97.2
3	5.5	61.8	7.6	nd	74.9*
4	5.4	61.7	7.5	16.0	90.6
Post-hydrolysis spike					
1	0.8	90.7	4.6	nd	96.1*
2	0.6	89.5	4.5	nd	94.6*
3	0.7	87.6	4.6	nd	92.9*
4	0.8	87.8	5.4	nd	94.1*

nd : not determined. * : excluding pellet.

The method described has four stages: deconjugation, extraction, defatting and analysis:

Deconjugation

Hormone esters (conjugates), principally glucuronides and sulphates, are present in liver due to metabolism. Determination of these conjugates is not important where it is required only to detect hormone use. Deconjugation of all such esters to the parent hormone prior to analysis simplifies the analysis and improves the detection limit because the residue is no longer distributed between a number of different conjugates. A number of methods for hydrolysis are available, although the normal approach is enzymolysis with the glucuronidase and aryl sulphatase activities present in *Helix pomatia* extracts. This technique is effective for phenolic estrogen hormones such as the stilbenes, but may be inadequate for steroid androgen sulphates [8]. *H. pomatia* extract was used in the work reported here but an investigation is currently in progress of the efficiency of enzyme and chemical methods for hydrolysis of a range of steroid glucuronide and sulphate esters.

Extraction

After deconjugation, the parent hormones were extracted from the tissue matrix using the three-phase partition method [5]. From radiolabel experiments, Henion and colleagues obtained 67% and 85% recoveries respectively for dexamethasone [5] and β–trenbolone [9] with this procedure. We found that the extraction efficiency for 19-*nor*testosterone depended upon the time of addition of the spike to the sample (Table 4). This explains the poor recovery for 19-*nor*testosterone shown in Table 3. If the spike was added immediately before extraction, recovery was effectively complete. Where the spike was added before hydrolysis (and therefore in contact with the sample for a period of about 16 hours) 15-20% of the activity was bound to the tissue in a form not extractable by the three phase system, but which could be solubilised with methanol. In addition, the distribution of activity between the three phases was different. The relatively low level of spiking for the radiolabelled experiments is unlikely to have influenced the result because recoveries for the three phase method were similar to those experienced at higher spiking concentrations with unlabelled 19-*nor*testosterone.

Liver tissue contains some functioning enzymes even after frozen storage and steroids spiked into the tissue could be subject to metabolic changes which would give rise to reduced recovery. Two possible routes are isomerisation of 17-hydroxy steroids between the α and β forms and also their esterification to the sulphate or glucuronide esters. Incubation of homogenised liver for 16 hours at 37 °C during *H pomatia* treatment may allow these processes to occur although, with regard to esterification, only the formation of sulphates will give rise to a problem because the *H pomatia* enzyme will cleave glucuronides. There is however insufficient evidence to decide whether these or other metabolic transformations are giving rise to the losses experienced.

Defatting

The mass of residue after evaporation of the middle phase was 1-2% of the initial sample weight, which presented problems applying solid phase extraction cartridge cleanup procedures. Low-resolution SEC was therefore applied to remove lipids and other co-extractives. The mobile phase employed was selected to minimise gel-analyte interactions and maintain as pure a size exclusion mechanism as possible. SEC achieved 90-95% recovery of radiolabelled hormone with 20-fold purification. This enables injection of 4 gram equivalents of liver onto the analytical coupled column system.

Analysis

The system consisted of three coupled columns, giving sequential GPC and normal phase HPLC separations on cyano and silica stationary phases. The overall configuration (Figure 1) was relatively complex because of the requirement to determine two compounds which were resolved on the GPC column. It is intended to automate the system fully.

GPC conditions were chosen for the initial coupled column separation to permit loading *ca* 5 mg of extract and also to provide a difference in selectivity from the SEC system used to defat the extract. During an early stage in development of the system, when 100% toluene (an SEC solvent) was employed as the mobile phase for the PLgel column and with subsequent transfer of analytes to a single Spherisorb S5W column, there was little difference in the trenbolone analysis, but 19-*nor*testosterone was inadequately resolved from interferences.

One problem with GPC is the potential for accumulation of sample components on the column. In particular, aromatic compounds are retained via $\pi-\pi$ interactions. There is some retention of trenbolone, compared to 19-*nor*testosterone, due to its conjugated double bond system. After extensive use, including the analysis of other extracts which were very dirty, a slow increase in PLgel column retention times was noted. Weekly flushing with toluene eliminated this problem. Initially the PLgel column was connected directly to the UV detector and trenbolone injections used to monitor the efficiency of flushing, but contamination of the flow cell occurred. Although this could be removed by purging it sequentially with propan-2-ol and then 2,2,4-trimethyl-pentane, the system required about 4 hours to restabilise. As no change in retention time has been noted in the 6 months since toluene purging was initiated, monitoring has been discontinued and the PLgel column simply flushed to waste with toluene for 2 hours each week. It is not possible to use direct UV detection to determine the retention time of 19-*nor*testosterone, because of absorbance from the solvent. For this compound PLgel column retention time information was obtained by making sequential injections of standards and trapping different fractions on the concentration column, with subsequent analysis by normal phase HPLC.

GPC fractions were refocussed on a silica reconcentration column before analysis on normal phase HPLC. A single normal phase column did not provide adequate resolution, so a second column switching stage was employed. Another normal phase column was chosen in order to simplify interfacing. Care was taken to select a complementary combination of columns. Cyano and diol phases were too similar. An aminopropyl phase was rejected because of interactions between the column and steroids, giving rise to reductions in peak areas of up to 75%. Reduced flow rate and stopped flow experiments showed that losses increased with contact time between column and analytes. C_1 and C_6 reversed phases, and strong cation and anion exchangers were all unsatisfactory, because of either too strong or too weak a retention. The silica phase actually used was unique in giving a different selectivity to the cyano column, as evidenced by inversion of retention order for trenbolone and 19-*nor*testosterone. Other types of silica examined, including a Spherisorb S5W column previously used for another analysis, gave inadequate resolution. However, the cyano/silica column set used has given stable and consistent results for several hundred injections over a period of one year.

In conclusion, the system is robust and reliable, with negligible loss of analyte during SEC defatting or coupled column HPLC analysis. The method affords high sensitivity, and permits extensive automation and application to other steroid hormones

possessing suitable UV chromophores. It could be applied to a wider range of steroids if extracts were derivatised before the coupled column separation.

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Received: May 20, 1992
Accepted: June 3, 1992