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Comparison and evaluation of the specificity and binding capacity of commercial and in house affinity columns used in sample preparation for analysis of growth-promoting drugs

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Abstract

Immunoaffinity chromatography (IAC) and affinity chromatography (AC) are widely used for extraction of drugs from biological samples. Fifteen column types were purchased from five different manufacturers and their ability to bind specific drugs including β-agonists and anabolic steroids over a range of analyte concentrations in fortified bovine urine samples was assessed. The performance data obtained from these columns were compared with columns produced in this laboratory (in house columns). The in house columns gave the highest recoveries, ranging from 92 to 100% at the 1 ng spiking concentration, for five of the seven analytes assessed. Forty percent (11 of 27) of all the commercial column assessments recorded recoveries of less than 50% even when the lowest spiking concentration was applied (1 ng). For one manufacturer, only one of seven different columns purchased delivered extraction efficiencies greater than 50%. The extraction efficiencies of the clenbuterol columns were the highest with all commercially prepared columns showing at least 50% binding of radiolabelled tracer. Recoveries of α-nortestosterone were the lowest. The variability of these products with respect to quality control requires constant monitoring.

Keywords: Growth-promoting drugs; Anabolic steroids; β-Agonists

1. Introduction

Residues of veterinary drugs have been detected in a wide array of biological samples. A number of different strategies have evolved to facilitate the concentration and purification of the compounds of interest. These have been based on solvent partitioning [1], solid-phase extraction [2], affinity chromatography [3] or combinations of these. Due to the health, safety and environmental problems associated with the storage, handling and disposal of organic solvents there has been a trend towards use of affinity procedures, especially immunoaffinity chromatography (IAC) (for review see Refs. [4,5]). The IAC technique is relatively straightforward but the production of the immobilised ligands is less so. Assuming highly specific antibodies are available, optimised protocols are necessary. To avoid these difficulties some laboratories rely upon commercial manufacturers to produce and validate IAC and affinity chromatography (AC) columns.

A number of other European laboratories (C. Elliott, personal communication) have reported difficulties with IAC sample preparation techniques

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using columns purchased from commercial sources. This laboratory has accumulated over eight years experience in IAC production and performance for veterinary drug extraction methods [6,7].

This study was undertaken to compare the performance of a number of commercial IAC and AC products with those produced in this laboratory. Analyte binding capacity was assessed by recovery of radio-isotopic anabolics from spiked bovine urine. The suitability of the extracts produced for analysis was not investigated due to the wide range of methodologies to which such extracts are currently applied [7–9].

2. Experimental

2.1. Apparatus

The instruments used were a centrifuge obtained from IEC (Needham Heights, MA, USA), Model Centra 8R and a magnetic stirrer obtained from Stuart Scientific (Redhill, UK), a pH meter (Corning Medical and Scientific Instruments, Halstead, UK), a waterbath (Grant, Cambridge, UK), a Uvimaster UV–Vis spectrophotometer (Pharmacia, St. Albans, UK), a vortexer (Fisons Scientific Equipment, Loughborough, UK), a roller mixer (Denley Instruments, Billingshurst, UK), an end-over-end mixer (Luckham, Sussex, UK), a vacuum pump (Gelman Sciences, Ann Arbor, MI, USA) connected to a manifold (J.T. Baker, Reading, UK). Radioactivity was monitored using a Wallac Rackbeta Spectal Cooled Counter Model 1219 (EG&G Berthold, Milton Keynes, UK).

2.2. Reagents and chemicals

IAC and AC columns were purchased from five commercial companies. A multi-analyte IAC (MIAC) column for zeranol (ZER), diethylstilbestrol (DES) and nortestosterone (NT) and a β-agonist column was purchased from company A whilst a MIAC for ZER/DES/NT, a single analyte IAC for trenbolone (TREN) and a β-agonist MIAC column were purchased from manufacturer B. Single analyte columns for NT, TREN, ZER, DES and clenbuterol (CBL) and a β-agonist column were purchased from manufacturer C. Manufacturer D supplied a TREN/NT, a stilbenes and a β-agonist column. The only column purchased from manufacturer E was an AC for β-agonists. All gels with the exception of those purchased from manufacturer E had a 1-ml bed volume. The bed volume of the columns from manufacturer E was not stated in the manufacturer's protocol.

All chemicals were obtained from BDH (Poole, UK) and were of Analar grade unless otherwise stated. Methanol and ethanol (HPLC grade) were obtained from Rathburn (Walkerburn, UK). Scintillation fluid (OptiPhase HiSafe) was purchased from Wallac (Turku, Finland).

The synthetic support 3M Emphase™ Biosupport Medium, disposable 5-ml polypropylene columns and porous polyethylene discs were purchased from Pierce and Warriner (Chester, UK).

Helix pomatia digestive solution (cat. No. G-0876) was obtained from Sigma (Poole, UK).

Radiolabelled tracers for 17β-nortestosterone (β-NT), trenbolone (TREN), zeranol (ZER), diethylstilbestrol (DES), clenbuterol (CBL), cimaterol (CIM)) were obtained from Amersham (Buckinghamshire, UK). 17α-Nortestosterone (α-NT) was bought from New England Nuclear (Boston, MA, USA). The tracers had specific activities of 53.6, 6.2, 45.0, 75.0, 9.8, 28.0 and 46.8 Ci/mmol, respectively.

Radiolabelled tracers were serially diluted in methanol until a working concentration of 15–20 000 dpm per 50 μl was achieved. The concentration of individual analytes in these tracer solutions ranged from 1 to 5 pg.

2.3. Buffer solutions

Coupling buffer (pH 9) contained equal amounts of 0.2 *M* sodium carbonate and 1.2 *M* sodium citrate. Quenching solution (pH 9) was prepared by diluting 18 ml of ethanolamine to 100 ml with deionised water. Wash solution contained 1 *M* NaCl and regeneration solution contained 4 *M* NaCl in 0.1 *M* phosphate buffer pH 7.2. Deconjugation solution was prepared by diluting *Helix pomatia* solution (1:25, v/v) in 0.2 *M* sodium acetate buffer (pH 5).

2.4. Antibody production and purification

Derivatives of ZER, DES, TREN, NT, CBL and CIM were conjugated to carrier proteins [10,11]. After polyclonal antisera was raised against these drugs and their derivatives in New Zeland White rabbits [12] the specificity of each individual antisera was determined [13].

Antisera were purified by ammonium sulphate precipitation [14] and the protein concentration was determined spectrophotometrically (280 nm). The conjugation routes, carrier proteins used and final protein concentration etc., in each purified antisera raised are shown in Table 1.

2.5. Immobilisation of antibody to support

The 3M Emphase Biosupport medium is composed of a methylenebisacrylamide–azlactone copolymer that is very hydrophilic and highly cross-linked. The azlactone functionality of the medium is used to attach the ligand to the support through stable covalent linkages. Each antibody was coupled to gel individually (10 mg antibody/ml gel) then gels combined at a 1:1 ratio to give the required MIAC. Coupling of purified immunoglobulins to the matrix was performed as described in the manufacturer's instructions. Briefly, coupling took place by introducing antibodies to the matrix in coupling buffer and allowing the resultant slurry to mix for 2.5 h at room temperature (RT). After the coupled gel was washed in PBS all unreacted binding sites were blocked by the addition of quenching buffer. The gels were then washed in wash solution prior to storage in 20% ethanol. Two separate in house multi analyte columns were prepared. The first contained antibodies to the anabolic agents ZER, DES, TREN and NT (VSD1) and the second antibodies to the β-agonists CBL and CIM (VSD2).

2.6. Packing of immunoaffinity columns

The ethanol storage solution was removed from the gel and replaced with deionised water before addition of the gel to the column (1 ml bed volume). The gel was conditioned by washing sequentially with 20 ml regeneration solution, 10 ml of 0.5 *M* NaCl solution, 10 ml of 0.25 *M* NaCl solution, 10 ml of 80% ethanol and finally 10 ml of degassed deionised water.

2.7. Determination of recovery, binding capacity and specificity

In all cases, commercial columns protocols were followed in accordance with manufacturer's instructions. Tables 2 and 3 outline the protocols used for in house and commercial columns.

Throughout the experiments a single composite bovine urine taken from an animal known to be free from anabolic/β-agonist treatment was used. All urine used to assess the steriod columns required deconjugation prior to application. If, as in the case of manufacturers A, B and D deconjugation was detailed in the protocol then this was followed. However where this was not the case (manufacturer C) the deconjugation procedure used in this laboratory was applied. In this process deconjugation solution was added to the urine (1:2, v/v) and the pH adjusted to 5±0.2 using 1 *M* acetic acid. After incubation overnight at 37°C deconjugation was considered complete and the pH was adjusted with 1

Table 1
Antisera produced for immobilization on immunoaffinity columns

Antiserum	Derivative	Carrier	Conjugation technique	Protein concentration (mg/ml)
ZER	ZER-carboxy methyl oxime	Human serum albumin	Mixed anhydride	10.4
DES	DES-carboxy propyl ether	Bovine thyroglobulin	Mixed anhydride	10.4
NT	NT-17-hemisuccinate	Bovine thyroglobulin	Mixed anhydride	13.0
TREN	TREN-17-Hemisuccinate	Transferrin	Mixed anhydride	11.3
CBL	DIAZO-CBL	Transferrin	Diazotization	7.4
CIM	DIAZO-CIM	Transferrin	Diazotization	9.6

Protein concentrations shown are those obtained after ammonium sulphate precipitation.

Table 2
Outline of in-house and commercial column protocols used during steroid column assessment showing pre column sample preparation, sample processing, elution procedures and column regeneration

VSD column 1	Manufacturer A	Manufacturer B	Manufacturer C	Manufacturer D
NT, DES, TREN, ZER MIAC	NT, DES, ZER MIAC	NT, DES, ZER MIAC, TREN IAC	NT, DES, TREN, ZER IACS	NT, TREN MIAC, DES IAC
Equilibrate column	Equilibrate column	Equilibrate column	Equilibrate column	Equilibrate column
Deconjugate urine	Deconjugate urine	Deconjugate urine	Deconjugate urine	Deconjugate TREN and NT urine only
Load urine (3 ml)	Load urine (0.25 ml). Leave 5 min	Load urine (0.25 ml). Leave 5 min	Load urine (3 ml)	Load urine (DES 3 ml, TREN/NT 4 ml)
Wash 3 ml 0.5 *M* NaCl	Wash 4 ml extraction buffer 1:10	Wash 4 ml extraction buffer 1:10	Wash 5 ml ethanol–distilled H_2O (10:90, v/v)	Wash 2×4 ml wash buffer (2×5 ml DES)
Wash 3 ml 0.25 *M* NaCl	Wash 5 ml distilled H_2O	Wash 5 ml distilled H_2O		Wash 5 ml distilled H_2O
Wash 1.5 ml distilled H_2O	Repeat both washes	Repeat both washes		
Add 1.5 ml 80% ethanol	Aspirate gently	Aspirate gently		
Elute 3.0 ml ethanol–distilled H_2O (80:20, v/v)	Add acetone–H_2O (95:5, v/v). Leave 5 min. Elute	Add acetone–H_2O (95:5, v/v). Leave 5 min. Elute	Elute 3 ml ethanol–H_2O (70:30, v/v)	Elute 3 ml ethanol–H_2O (70:30, v/v)
Elute 3.0 ml ethanol–distilled H_2O (80:20, v/v)	Wash acetone–H_2O (95:5, v/v)		Rinse 3 ml ethanol–H_2O (80:20, v/v)	Wash methanol–H_2O (70:30, v/v)
Wash 3 ml distilled H_2O	Wash 10 ml distilled H_2O		Wash 10 ml PBS	Equilibrate 15 ml wash buffer 2

Table 3
Outline of in-house and commercial column protocols used during β-agonist column assessment showing pre column sample preparation, sample processing, elution procedures and column regeneration

VSD column 2	Manufacturer A	Manufacturer B	Manufacturer C	Manufacturer D	Manufacturer E
β-Agonist column	β-Agonist column	β-Agonist column	CBL column, β-agonist column	β-Agonist column	β-Agonist column
Equilibrate column	Equilibrate column	Equilibrate column	Equilibrate column	Equilibrate column	Equilibrate column
	Deconjugate	Deconjugate			
Load urine (3 ml)	Load urine (0.25 ml). Leave 5 min	Load urine (0.25 ml). Leave 5 min	Load sample (3 ml)	Load sample (4 ml)	Load urine (1 ml)
Wash 3 ml 0.5 *M* NaCl	Wash 8 ml extraction buffer 1:10	Wash 8 ml extraction buffer 1:10	Wash 5 ml distilled H_2O	Wash 2×4 ml wash buffer	Wash 2 ml 50 m*M* KH_2PO_4 buffer pH 3.0
Wash 3 ml 0.25 *M* NaCl	Wash 2 ml extraction buffer 1:100	Wash 2 ml extraction buffer 1:100		Wash 5 ml distilled H_2O	
Wash 1.5 ml distilled H_2O					
Add 1.5 ml 80% ethanol	Aspirate gently	Aspirate gently			
Elute 3.0 ml ethanol–0.2 *M* sodium acetate buffer pH 4 (80:20, v/v)	Add ethanol–acetic acid (96:4, v/v) pH 3.5–4.0. Leave 5 min. Elute	Add ethanol–acetic acid (96:4, v/v) pH 3.5–4.0. Leave 5 min. Elute	Elute 3 ml ethanol– (0.5 *M* NaCl: 0.58%) acetic acid (50:50, v/v) pH 3.5	Elute 4 ml ethanol–H_2O (70:30, v/v) pH 5	Elute 1 ml methanol–H_2O (50:50, v/v)
Wash 3.0 ml ethanol–0.2 *M* sodium acetate buffer pH 4 (80:20, v/v)	Wash 10 ml ethanol–acetic acid (96:4, v/v) pH 3.5–4.0	Wash 10 ml ethanol–acetic acid (96:4, v/v) pH 3.5–4.0	Rinse 3 ml ethanol–H2O (80:20, v/v)	Add 15 ml elution buffer	Discard column
Wash 3 ml distilled H_2O	Wash 10 ml distilled H_2O	Wash 10 ml distilled H_2O	Wash 10 ml PBS	Equilibrate with 20 ml wash buffer	

M sodium hydroxide to pH 10±0.2. Deconjugation of urine for assessment of β-agonist columns was required only by manufacturers A and B and was carried out as detailed in the protocols provided. The remaining β-agonist columns were assessed using untreated urine.

The required volume of the appropriate urine was spiked with a known quantity of radiolabelled tracer and applied to the column to assess recovery. Increasing concentrations (1 to 200 ng) of unlabelled drug were then added to the urine samples to assess column binding capacity. All eluates (i.e., sample supernatants, washes and the final eluates which were expected to contain the specifically bound analyte) from columns were collected separately and the radioactivity measured in each.

2.8. Interpretation of data

The percentage of radiolabelled tracer recovered in each eluate was determined for every column. It was assumed that the percentage of radiolabelled material recovered was an accurate estimation of the percentage of unlabelled analyte also recovered in each eluate. Data were normalised against the highest percentage recovery recorded when tracer only (50 μl) was added (i.e., column binding capacity for 1 to 5 pg analyte). The ability of each column to yield 50% recoveries of added analyte in the final eluate was assessed by plotting percentage recoveries against analyte concentration and interpolating the 50% recovery value. This value has been expressed as the R_{50}.

3. Results and discussion

Column performances with respect to the analyte concentrations which gave 50% recoveries have been summarised (Table 4). As a guide to the capacity of each column to bind analyte the distribution of radiolabel contained within the various eluates collected at the 5-ng column loading level have been detailed in Table 5.

Of the five NT columns assessed using β-NT two (D and VSD) showed consistent recoveries in excess of 80% throughout the concentration range (0–200 ng). Recoveries recorded from the remaining three columns (A, B, C) were 54, 35 and 20% respectively at a 10-ng loading level (Fig. 1). The two columns which had performed best with β-NT also recorded the highest recovery with the α-NT (93 and 72% at 10 ng). The remaining columns however showed practically no binding of this epimer (4, 2, 2% for A, B and C) (Fig. 2). Two of the four TREN columns (VSD and D) were very effective. These columns showed R_{50} values of >200 ng. The remaining TREN columns (B and C) did not achieve R_{50} values even with the addition of only 1 ng of unlabelled analyte (42 and 40%) (Fig. 3). One commercial ZER column (C) gave unsatisfactory recoveries (7% on the addition of 10 ng analyte). Percentage recoveries from the remaining ZER columns at 10 ng were 64,

Table 4
R_{50} values calculated for each column

Manufacturer	Analyte						
	β-NT	α-NT	TREN	DES	ZER	CBL	CIM
VSD	>200	122	>200	>200	>200	>200	100
A	24	0*	N/A	52	127	100	N/A
B	2.5	0*	0*	0.02	152	126	N/A
C	0*	0*	0*	0*	0*	34	N/A
C#	N/A	N/A	N/A	N/A	N/A	1.27	0*
D	>200	>200	>200	>200	N/A	>200	0.77
E	N/A	N/A	N/A	N/A	N/A	>200	>200

The R_{50} value is the concentration of cold drug (ng) which when added to the column results in a 50% recovery of radiolabel.
0* Denotes that at no stage was 50% binding detected.
N/A Denotes that a particular column was not available from a manufacturer.
Values of >200 were recorded when the addition of 200 ng of unlabelled drug failed to reduce binding to 50%.

Table 5
Percentage tracer recovered during the various stages of sample preparation when 5 ng of unlabelled drug was applied to the column

Analyte	Manufacturer	% Recovery			
		Step 1	Step 2	Step 3	Step 4
ZER	A	14.3	16.8	62.2	6.7
	B	12.5	17.4	52.2	17.9
	C	17.2	45.0	9.4	28.4
	VSD	4.2	19.1	75.7	1.0
DES	A	6.6	26.0	50.9	16.5
	B	2.4	9.4	25.6	62.6
	C	2.0	9.4	35.8	52.8
	D	1.5	5.8	92.7	0
	VSD	1.1	5.3	80.8	12.8
α-NT	A	43.4	53.1	3.5	0
	B	39.2	56.0	2.7	2.1
	C	19.4	45.0	4.2	31.4
	D	1.8	5.8	72.9	19.5
	VSD	0.9	7.0	85.0	7.1
β-NT	A	21.6	42.1	35.9	0.4
	B	19.4	35.7	29.5	15.4
	C	25.4	45.3	15.1	14.2
	D	8.0	18.8	63.2	10.0
	VSD	4.5	17.4	67.8	10.3
TREN	B	10.4	17.8	23.2	48.6
	C	13.3	12.3	27.7	46.7
	D	7.3	17.6	58.7	16.4
	VSD	5.7	18.3	65.2	10.8
CBL	A	3.9	5.1	86.5	4.5
	B	3.8	3.9	84.3	8.0
	C	11.6	14.5	59.4	14.5
	C*	24.4	39.6	18.7	17.3
	D	2.7	5.6	47.1	44.6
	E	1.9	3.5	73.8	20.8
	VSD	3.5	7.5	85.9	3.1
CIM	C*	36.4	52.9	6.3	4.4
	D	7.0	10.2	36.2	46.6
	E	6.5	6.4	79.8	7.3
	VSD	6.7	10.5	75.0	7.8

Step 1 shows the % tracer which passed straight through the column during sample application; step 2: tracer recovered during the wash step(s); step 3: % tracer in final eluant (analyte specifically bound); and step 4: tracer which remained unaccounted for throughout the assessment and was assumed to have remained bound to the column.
C* = β-agonist column.

84 and 100% for A, B and VSD respectively (Fig. 4). Three of the five DES columns showed recoveries above 90% after the addition of tracer only (VSD 92%, A 100%, D 99%), however one of these columns (A) showed a decrease in binding of 36% when 1 ng of unlabelled drug was added. At the 10 ng loading level the recoveries recorded were 94, 99, 65, 42 and 28% for VSD, D, A, C and B respectively (Fig. 5). The CBL columns performed satisfactorily. Only one column (β-agonist column produced by C) recorded a recovery of less than 45% after the addition of 5 ng of unlabelled drug. Other recoveries achieved at this level were A 104%, B 101%, C (CBL only column) 72%, D 57% and E 89% (Fig.

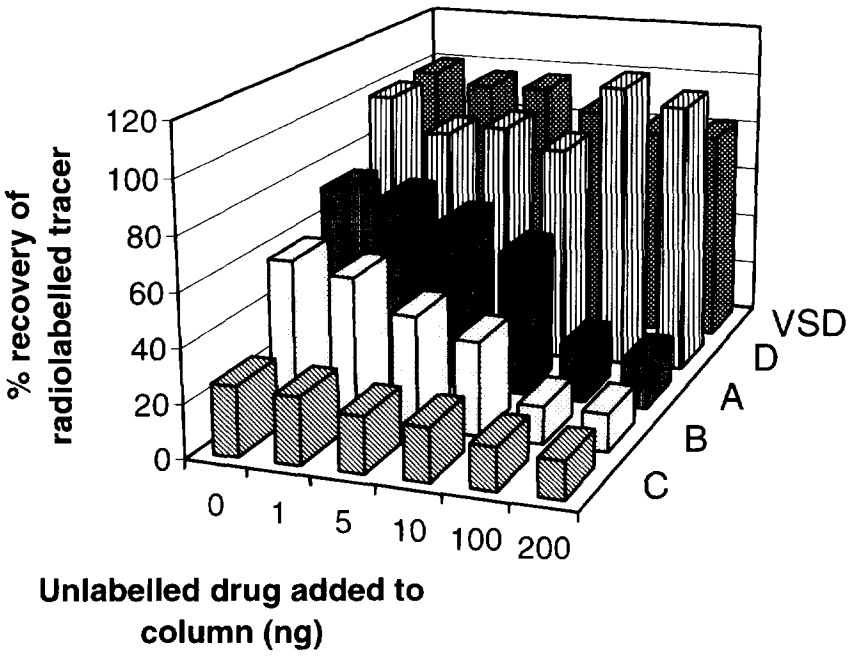

Fig. 1. Recovery of β-NT from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

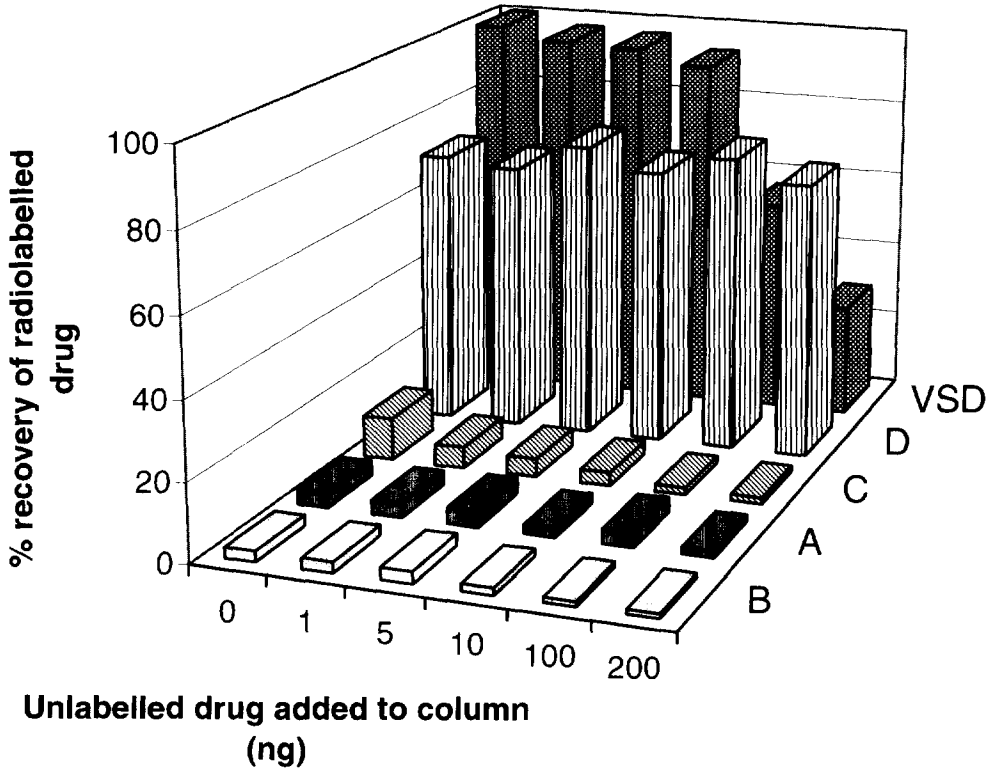

Fig. 2. Recovery of α-NT from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

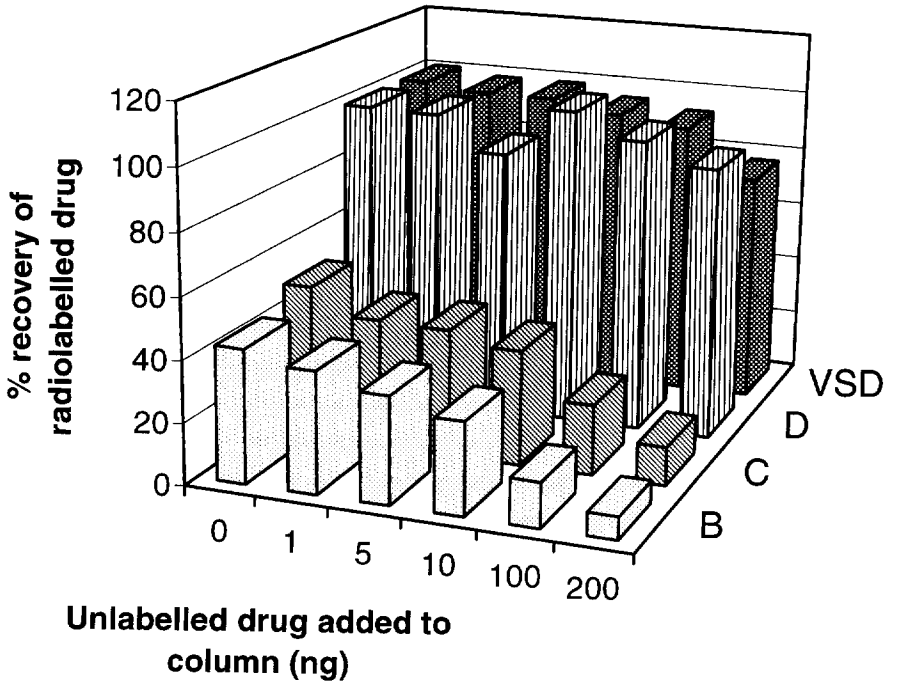

Fig. 3. Recovery of TREN from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

6). Of four expected to capture CIM only two columns gave R_{50} values of greater than 10 ng; both VSD and E showed 92% binding at this loading. A third column (D) showed only adequate binding over the concentration range (45% at 10 ng) whilst the fourth column (C) did not achieve R_{50} at any loading level (6% at 10 ng) (Fig. 7).

This assessment of commercial affinity columns for sample preparation has identified large differences in the recoveries of analyte from spiked bovine

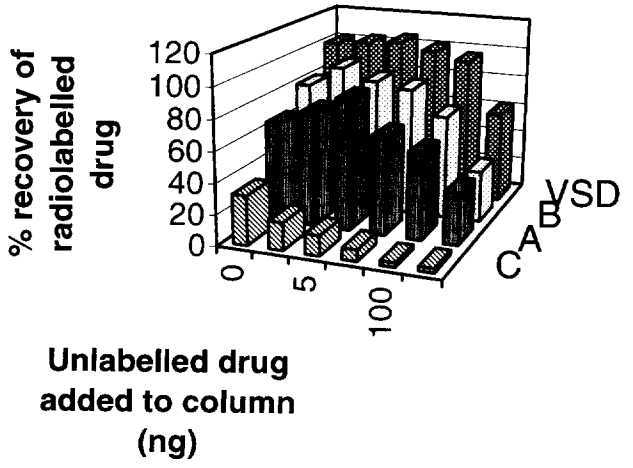

Fig. 4. Recovery of ZER from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

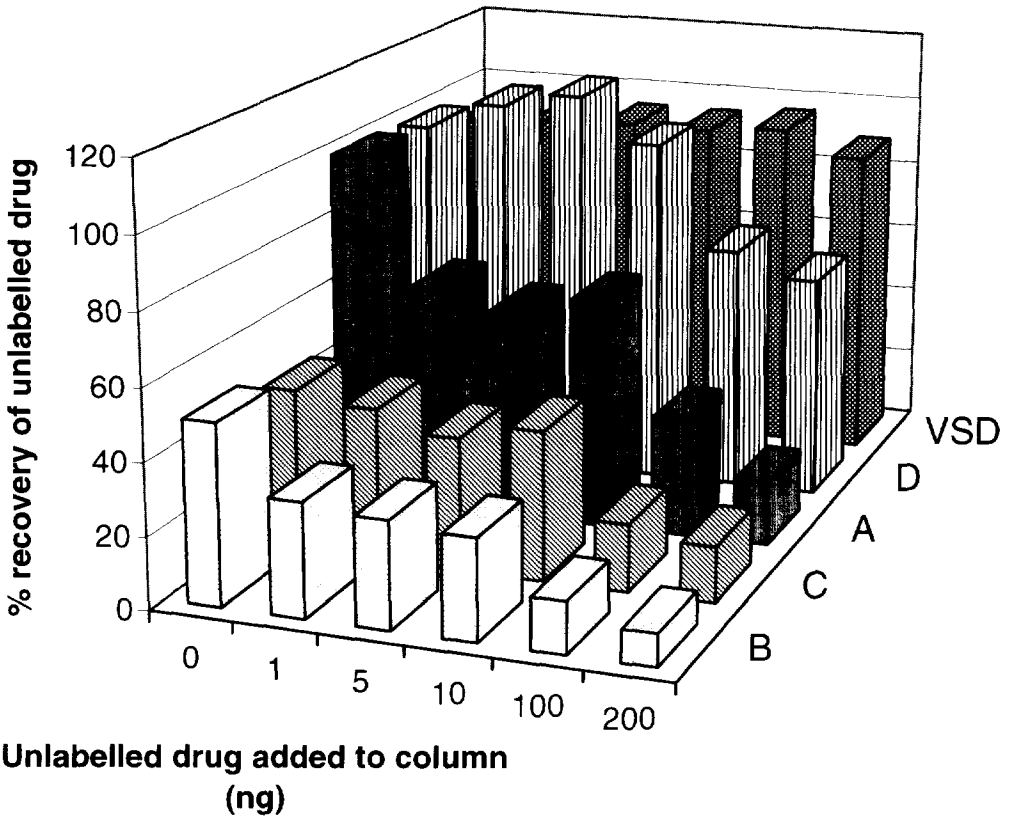

Fig. 5. Recovery of DES from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

urines. Previous experience with IAC in this laboratory (unpublished data) suggests that there are at least three possible reasons for the failure of columns to adequately entrap and then elute the compound of interest.

The first possible problem is poor antibody specificity. This is identified by failure of columns to adequately entrap the analyte(s) of interest or by entrapment of unrelated compounds. In this study the antibody on some NT columns for example, recommended for use with urine, did not recognise the antigen. In one case when α-NT, the major urinary metabolite of nortestosterone esters such as nandrolone and laurabolin [15,16], was added to the column 96% was not bound (Fig. 2). This same column did bind the β-epimer of the drug (Fig. 1), the minor urinary metabolite, thus indicating that a β-specific NT antibody had been incorporated into the gel.

It is possible that a similar problem with antibody specificity was demonstrated during entrapment of CIM. The β-agonist column produced by C showed lower binding of CBL than the column recommended for CBL only (Fig. 6). Binding of CIM by the β-agonist column (Fig. 7) was also limited (<50% at all levels). The need to entrap multi β-agonists may have resulted in the inclusion of less specific antibodies which although showing some level of entrapment may have reduced binding capacities in some cases to an unacceptable level.

A second area of poor performance is insufficient antibody concentration or antibodies of poor avidity creating an imbalance between the binding capacity of the column and the quantity of analyte to be bound. This is identified when high recoveries are achieved initially with low concentrations of analyte but fall rapidly as analyte concentration is increased. For example addition of 1 ng of unlabelled DES to a

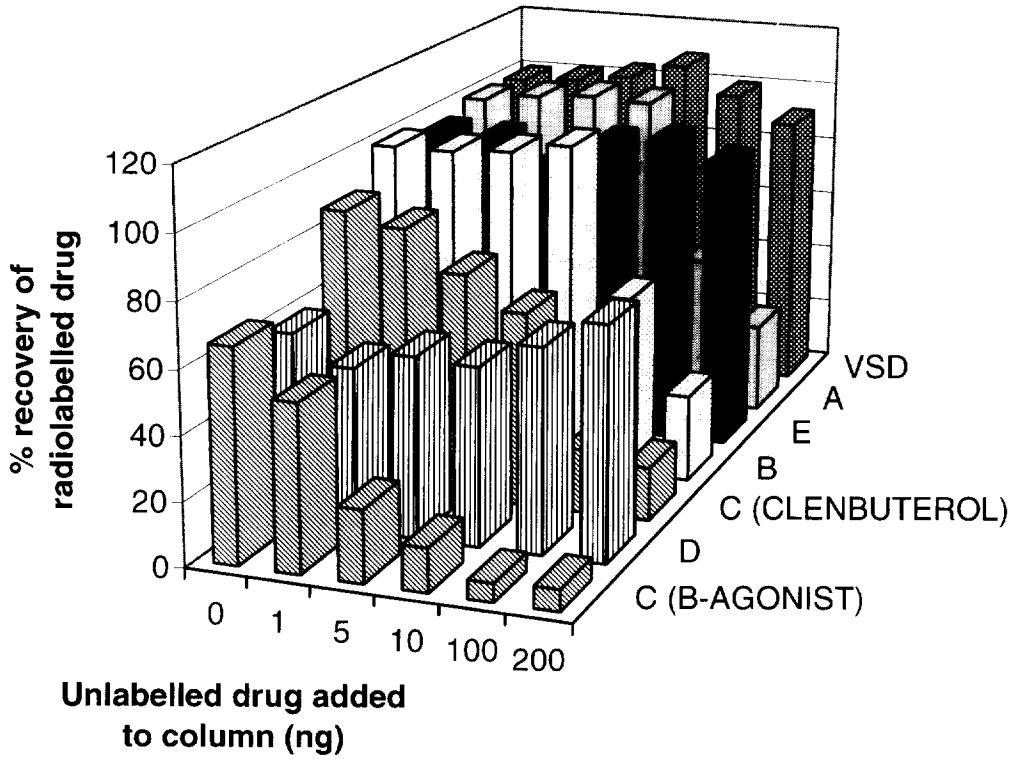

Fig. 6. Recovery of CBL from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

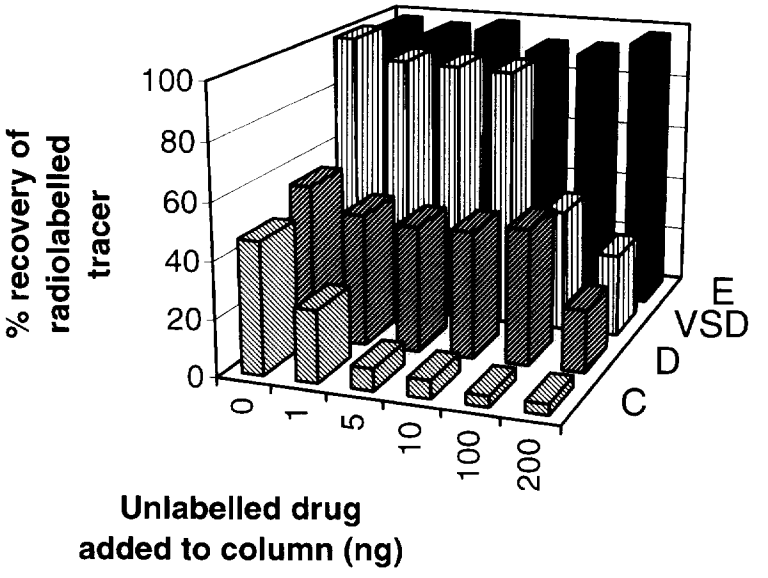

Fig. 7. Recovery of CIM from the affinity columns produced in house and those purchased after addition of unlabelled drug (0–200 ng).

column was shown to reduce binding by up to 36% (Fig. 5). DES concentrations in bovine urine up to 500 ng/ml have been detected after intramuscular injection of DES dipropionate [17].

Thirdly problems with some columns occur because of inadequately defined protocols. In this study tracer was retained in many columns after the recommended elution. This may be due either to excess antibody on the column or to the elution steps being inadequate. In the in house columns excess antibody has been shown to significantly retard analyte elution (data not shown). In the commercial columns up to 18% of the CBL, unaccounted for after elution was detected in the regeneration wash(es). Although the CBL column which showed the highest concentration of radiolabelled tracer in the regeneration wash was not recommended for use with urine it is unlikely the urine adversely affected the elution efficiency.

There may be other reasons for the data demonstrated in the present study. The results have however, highlighted the need for affinity column users to ensure that column performance matches the required assay sensitivity and for column manufacturers to maintain stringent internal quality control programmes.

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