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MULTI-IMMUNOAFFINITY CHROMATOGRAPHY: A SIMPLE AND HIGHLY SELECTIVE CLEAN-UP METHOD FOR MULTI-ANABOLIC RESIDUE ANALYSIS OF MEAT

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SUMMARY

A method for the detection of nortestosterone (NT) in bovine muscle at levels below 1 μg/kg is described, based on enzymatic digestion of the sample, clean-up by immunoaffinity chromatography after defatting and detection by gas chromatography–mass spectrometry (selected-ion monitoring). The immunoaffinity matrix was prepared after combining the isolated immunoglobulin G fractions from a rabbit antiserum raised against NT and methyltestosterone (MT). Its capacity per millilitre of gel was approximately 10 ng for each of the two steroids. Results for samples containing 0.1 μg/kg NT and above are described. It is concluded that for multi-residue analysis of samples of muscle at levels as low as 0.1 μg/kg, multi-immunoaffinity chromatography is a very suitable method of sample clean-up. For purposes of quantification the trideuterated internal standard [16,16,17α-$^2H_3$]nortestosterone was synthesized.

INTRODUCTION

For many years investigators have worked on the development of methods for the detection of residues of anabolics in biological samples. However, only a limited number of methods which allow the detection at levels below 1 μg/kg (sub-ppb) in muscle have been reported. For example, Meyer and Hoffmann [1] reported an enzyme immunoassay for trenbolone, Van Peteghem [2] a chemiluminescence immunoassay for 19-nortestosterone and Van Peteghem and Van Haver [3] a radioimmunoassay for diethylstilbestrol. Facino et al. [4] reported a tandem mass spectrometric method for the detection of diethylstilbestrol, zeranol and trenbolone. With the exception of the last method, all these procedures involve a high-performance liquid chromatographic (HPLC) purification step.

Detection techniques used in on-line combination with HPLC usually lack the sensitivity for detecting anabolics in picogram amounts, making combination with

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a separate detection technique necessary. Unfortunately, this hampers the development of multi-residue methods. A possible alternative to HPLC is immunoaffinity chromatography (IAC). This technique has been used successfully in the determination of low levels of estradiol in serum [5], chloramphenicol in muscle [6] and trenbolone in urine [7].

For methods to be applied to very low residue levels, the recovery of the procedure is of paramount importance. De Groot and Wubs [8] used a mixture of collagenase and subtilisin Carlsberg, which was incubated, in the presence of an antibiotic-containing solution, with muscle for 8–15 h under physiological conditions of temperature and pH. Van Peteghem [2] and Cordonnier et al. [9] used a suspension of the protease subtilisin A, a heat-stable enzyme. These digestion procedures resulted in high recoveries of the analytes studied.

The aim of this investigation was to develop a reliable gas chromatographic–mass spectrometric (GC–MS) method for the detection of residues of anabolics with IAC purification and enzymatic digestion of the sample. Here we report the preparation of a multi-immunoaffinity (MIAC) matrix suitable for nortestosterone (NT) and methyltestosterone (MT). For purposes of quantification, the internal standard [16,16,17α-$^2H_3$]nortestosterone (NT-d3) was synthesized.

EXPERIMENTAL

Chemicals

All solvents, reagents and chemicals were of analytical-reagent grade from Merck[a] (Darmstadt, F.R.G.), unless stated otherwise.

Antisera and standards

The deep-frozen antisera used were raised in rabbits and were directed to the following immunogens: 19-nortestosterone-3-carboxymethyloxime–bovine serum albumin (19NT-3CMO–BSA) [10] and 17α-methyltestosterone-3-carboxymethyloxime–BSA (17αMT-3CMO–BSA) [11, 12]. The standards of NT and its 17α-epimer (epiNT) were a gift from Organon (Oss, The Netherlands). All other steroid standards used were from Sigma (St. Louis, MO, U.S.A.). All standards were checked for purity by mass spectrometry, Fourier transform infrared spectroscopy and HPLC with UV spectrum detection.

Internal standard, [16,16,17α-$^2H_3$]nortestosterone

The synthesis of the internal standard was performed in five steps using NT as the starting material. The 3-keto group of NT was protected as the diethylene ketal using pyridinium *p*-toluenesulphonate (PPTS) as a catalyst. A mixture of 2 g of NT, 2 ml of ethylene glycol and 1 g of PPTS was refluxed in 400 ml of benzene with azeotropic removal of water. The reaction products (1.2 g) were

[a]Reference to a company and/or product is for purposes of information and identification only and does not imply approval or recommendation of the company and/or the product by the National Institute of Public Health and Environmental Protection, to the exclusion of others which may also be suitable.

oxidized with 7 equiv. of pyridinium dichromate (PDC) in 100 ml of dimethylformamide, yielding the corresponding ketones. The reaction was quenched with 200 ml of water and the products were extracted with three 100-ml portions of diethyl ether. The mixture of ketones obtained was dissolved in dioxane (50 ml), and 30 ml of ^2H_2O (> 99% ^2H) were added followed by 3 g of K_2CO_3. The mixture was refluxed for 24 h and evaporated. The exchange was repeated using fresh dioxane and ^2H_2O. After evaporation, the product was dissolved in 100 ml of dichloromethane, 3 g of $MgSO_4$ were added and the mixture was stirred for 15 min. After filtration, the solvent was removed under reduced pressure giving 1.2 g of deuterated material. Reduction with 0.5 g of aluminium lithium deuteride in 50 ml of tetrahydrofuran gave a mixture of tetradeuterated hydroxyketals. Without isolation of these compounds the mixture was deketalized using 1 g of PPTS in refluxing wet acetone. During this reaction the 17-O^{2}H was exchanged for 17-OH. Pure NT-d3 was obtained by flash chromatography followed by crystallization from diethyl ether. According to mass spectrometry the NT-d3 contained 10% of dideuterated material.

Gas chromatography–mass spectrometry

All GC–MS analyses were carried out on a Finnigan 4500 (San Jose, CA, U.S.A.) quadrupole gas chromatograph–mass spectrometer. The Finnigan 9610 gas chromatograph contained a DB-1 fused-silica column (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm (J & W Scientific, Falsom, U.S.A.). The end of the column was passed through the separator oven and inserted directly into the mass spectrometer transfer line. Helium was used as the carrier gas at a column head pressure of $1.1 \cdot 10^5$ Pa. Splitless injections were performed. The GC oven temperature was increased ballistically from its initial temperature of 150 to 270°C 1 min after injection. The injection port, separator oven and transfer line temperature were held isothermally at 260, 260 and 270°C, respectively.

The mass spectrometer was operated in the negative-ion chemical ionization mode at an ion source temperature of 140°C and with a filament current of 200 μA. Methane was used as the reagent gas and the ion source pressure was maintained at 50 Pa. The electron energy varied between 70 and 90 eV. Data were acquired in the selected-ion monitoring (SIM) mode, using a dwell time of 0.052 s.

Preparation of the immunosorbent

The immunoglobulin G (IgG) fractions were isolated from the antisera with protein A coupled to Sepharose (Pharmacia, Uppsala, Sweden) and the protein content of the fractions was determined by the method of Lowry et al. [13]. The binding capacities for the different compounds were determined using Scatchard analysis with a computer program developed by Rodbard and Lewald [14]. The IgG fractions, when applicable, were mixed in such a way that the amounts of specific IgG were equal and the protein content was 1 mg IgG per ml. The antibodies were coupled to Tresyl-activated Sepharose® (Pharmacia) according to the supplier's instructions.

General assay protocol

To 1 g of homogenized (chopped) muscle was added the internal standard solution, containing 0.5 ng of NT-d3 in 0.05 ml of ethanol. The samples were digested by incubation in 4 ml of 0.1 mol/l. Tris buffer (pH 9.5) containing 1 mg of subtilisin A (Sigma) for 2 h at 60°C. The digestion was terminated by cooling the mixture and subsequent extraction with two 5-ml portions of diethyl ether, and the combined diethyl ether was removed under a stream of nitrogen [2].

Prior to the IAC the crude extract has to be defatted. Several methods have been shown to be suitable, e.g., the procedure described by Van Peteghem [2] in which the residue is dissolved in *n*-hexane–dichloromethane (85:15, v/v) and chromatographed on a column (0.5 cm I.D.) filled with a 6-cm layer of Lipidex 5000 (Packard, Groningen, The Netherlands). Chromatography on a Florisil SPE cartridge (J.T. Baker, Phillipsburg, NJ, U.S.A.) can also be used, sample application and washing with 1% (v/v) ethanol in light petroleum (b.p. 40–60°C) and elution with 20% (v/v) ethanol in light petroleum. The dry, defatted residue obtained after evaporating the eluting solvent was dissolved in 1.0 ml of 10% (v/v) aqueous ethanol. The MIAC columns, each containing 1 ml of gel, were washed with 10 ml of 0.5 mol/l phosphate-buffered saline (pH 7.5) and with 2 ml water. The sample was applied to the column, which was subsequently washed with two 2-ml portions of 10% (v/v) aqueous ethanol. The bound anabolic steroids were eluted with 2 ml of 50% (v/v) aqueous ethanol. The columns were regenerated by subsequent washing with 10 ml of 90% (v/v) aqueous ethanol, 5 ml of water, 5 ml of 0.1 mol/l sodium acetate (pH 4.0) and 5 ml of phosphate buffer, after which they were stored at 4°C.

The analyte fraction, after removing the ethanol under a stream of nitrogen, was extracted with two 4-ml portions of diethyl ether. The residue was subjected to derivatization with 0.025 ml of heptafluorobutyric anhydride (HFBA)–acetone (1:4, v/v). Under these conditions only NT-diHFB is formed in detectable amounts. To the crude reaction mixture of 0.4 ml of isooctane and 0.05 ml of acetonitrile were added. After mixing and separation of the layers, the isooctane layer was removed and the remaining solvent was extracted with 0.3 ml of isooctane. The isooctane fractions were combined in a Varian vial and the solvent was evaporated. The residue was dissolved in 25 μl of isooctane. For GC–MS analysis 1 μl was injected using a falling-needle injector. Prior to the injection a number of standard solutions were analysed, containing each a fixed amount of the internal standard (I.S.) and various amounts of, e.g., NT, similar to those expected in the samples. Each standard solution was analysed in duplicate and the responses (A) at the masses (m/z) 450, 469, 646 and 649 (I.S.) were recorded. The intensity ratios A_{646}/A_{649} were used to construct the isotope-dilution (ID) calibration graph (see also Results), whereas the intensity ratios A_{450}/A_{646} and A_{469}/A_{646} of the corresponding diagnostic ions were each averaged and used as identification criteria.

The GC–MS criteria were as follows. The retention time of a component should be equal to that of the standard (deviation less than 0.1 min or 0.5%, whichever is the smaller). The deuterated internal standard should be present and its response (A_{649}) should not be more than 1.2 or less than 0.4 times the average value

for A_{649} in the standard solutions. The ratios A_{450}/A_{646} and A_{469}/A_{646} should be within the range of ± 2 standard deviations (S.D.) of the mean value obtained for the standards. It should be noted that it is valid to calculate A_{450}, A_{469}, A_{646} or A_{649} only if the response maximum is clearly higher than the background.

RESULTS

Preparation and characterization of the immunosorbent

The antisera raised against NT and MT were characterized by a radioimmunoassay (RIA) procedure and subsequent Scatchard analysis. Table I shows the results and the calculated capacities (ng/ml) of the sera used. The IgG fraction obtained after protein A chromatography contained 2.15 mg/ml protein for the anti-NT serum and 2.25 mg/ml for the anti-MT serum (7-ml fractions). In a first experiment an individual matrix (IAC) was prepared for both NT and for MT. As an example, Fig. 1 shows antibody dilution curves for the original antiserum (anti-MT), for the IgG fraction obtained prior to the coupling reaction and for the supernatant of the reaction mixture after the reaction with the activated matrix. Fig. 1 shows a small loss of binding activity due to the protein A chromatography. After coupling, the fraction shows only little activity. To each millilitre of gel approximately 1 mg of the isolated protein (IgG) was coupled,

TABLE I

CHARACTERIZATION OF THE ANTISERA USED FOR THE MIAC MATRIX

Values based on a best-fit Scatchard analysis (two-parameter model).

Batch code	K_a (l/mol)	Binding capacity (mol/l)	Theoretical capacity (ng/ml serum)
H152458(anti-MT)[a]	$0.43 \cdot 10^{10}$	$0.11 \cdot 10^{-9}$	2050
H154213(anti-NT)[b]	$5.6 \cdot 10^{10}$	$0.78 \cdot 10^{-9}$	2000

[a]Final dilution (RIA), 1:60 000 (v/v).
[b]Final dilution (RIA), 1:9600 (v/v).

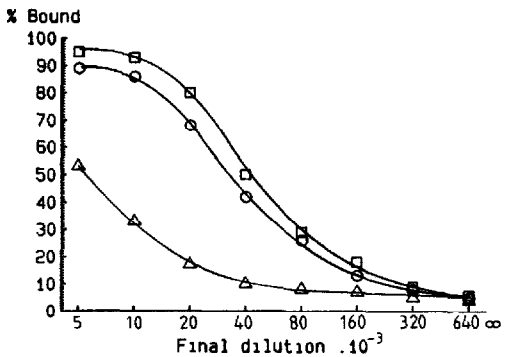

Fig. 1. Antibody dilution curves obtained by RIA for MT. □, MT antiserum (H 152458); ○, corresponding IgG fraction prior to coupling; △, corresponding IgG fraction after coupling.

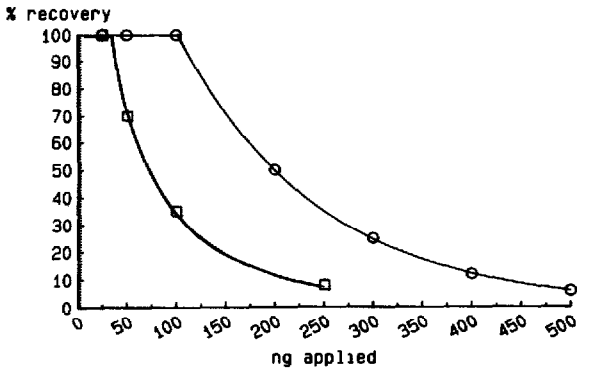

Fig. 2. Capacities of (○) the MT- and (□) the NT-IAC matrices as a function of the amount (ng) of MT and NT applied, respectively.

corresponding to ca. 5% of the total amount isolated. The calculated binding capacity is therefore approximately 100 ng/ml of gel.

The elution conditions for the matrices obtained were studied with the aid of radioactively labelled standards, [^{3}H]NT and [^{3}H]MT. Small amounts (300 Bq) were applied to the columns and the eluate was fractionated in 1-ml portions. The ethanol content of the eluting solvent was increased stepwise. The activity of the fractions was monitored with a liquid scintillation counter (LS300, Beckman, Fullerton, CA, U.S.A.) using Optifluor (Packard, Downers Grove, IL, U.S.A.) as the scintillation cocktail. With 50% (v/v) aqueous ethanol it was possible to elute both [^{3}H]MT and [^{3}H]NT quantitatively. Under these conditions the capacity of the columns containing 1 ml of gel was tested. Increasing amounts of standard NT and MT were applied to the column and the fraction which eluted with 50% (v/v) ethanol was analysed for the amount of NT or MT present by HPLC with on-line UV detection (254 nm). Fig. 2 shows the results of this experiment. When the recovery was less than 100% the amount lost always was present in the earlier fractions, indicating that the amount applied exceeded the capacity of the column. For MT the capacity was found to be 100 ng/ml and for NT 35 ng/ml of gel. For MT this agrees very well with the estimated value; for NT the result is slightly lower, but in view of the assumptions made during the capacity calculations and experimental uncertainties both results are satisfactory.

In a second experiment both IgG fractions were mixed prior to the coupling reaction. As for the coupling experiments in which only one kind of specific IgG was used, the experimentally found capacities of the MIAC gel were in good agreement with the calculated values. As the individual IgGs were mixed in equal amounts and the total amount was kept constant, a ca. 50% lower capacity for each steroid was calculated and found.

Analysis of muscle

Fig. 3 shows the mass spectra of NT and NT-d3, both as diHFB derivatives. The procedure was tested for the occurrence of false-positive results by analysing

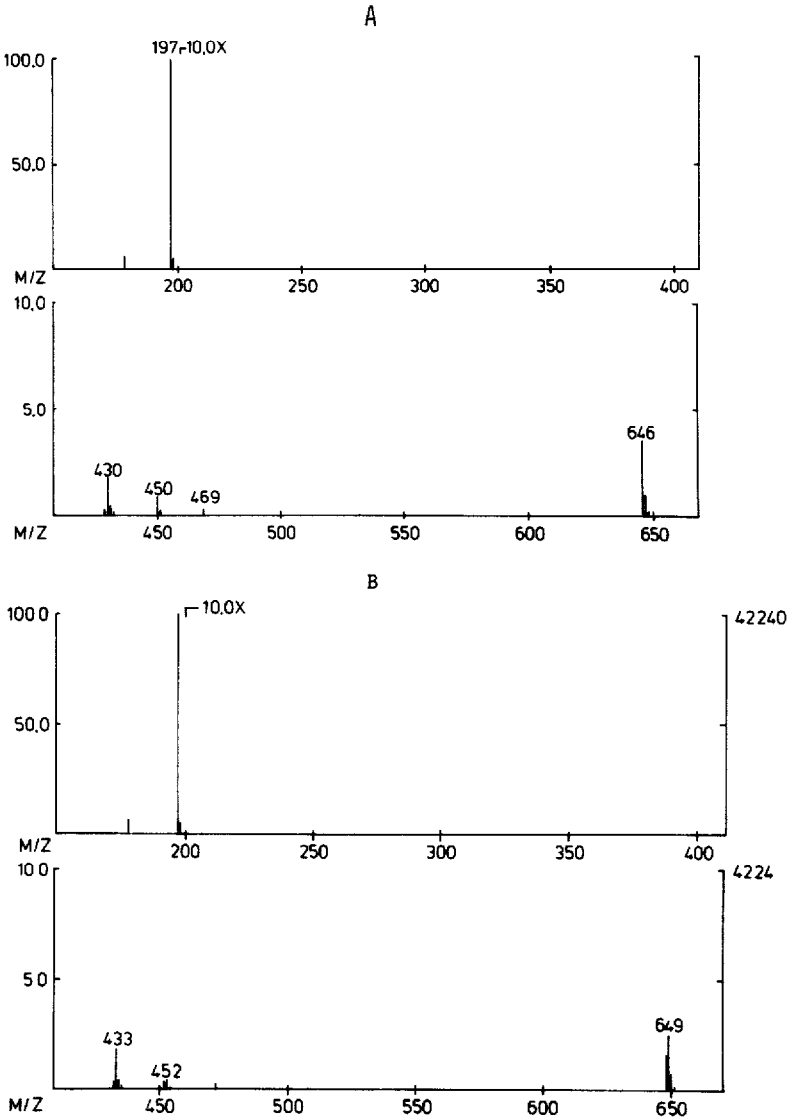

Fig. 3. Mass spectra of NT (A) and the internal standard NT-d3 (B) obtained by negative-ion chemical ionization.

representative blank samples known to originate from animals that had not been treated with any anabolic compound ($n=18$), including a series of fifteen samples obtained from the Dutch State Institute of Quality Control of Agricultural Products (RIKILT, Wageningen, The Netherlands). None of these samples contained responses that satisfied the criteria described. One of these samples was used to prepare a series of samples spiked with NT standards over the range 0.1–1.0 μg/kg. When the response ratio A_{646}/A_{649} for the spiked samples was used to construct an ID calibration graph (an 'internal' graph in contrast to an 'external' graph obtained by directly derivatizing and analysing the standards), it resulted

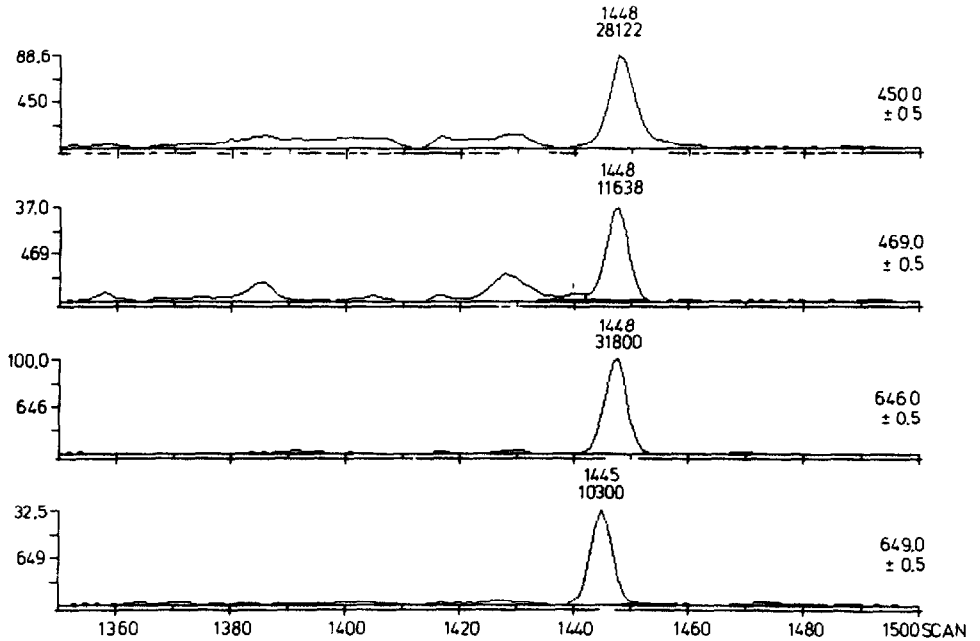

Fig. 4. Response of the selected ions for a sample containing 1.5 μg/kg NT spiked with 0.5 μg/kg NT-d3.

in a straight line passing through the origin. The slope of the internal calibration graph was identical with the slope of the external graph. For this reason it is not necessary to construct an internal calibration graph in each experiment, provided that a sufficient number of negative and positive control samples are analysed each time. The recovery of 0.5 μg/kg NT-d3 calculated for the response of the m/z 649 ion was $80 \pm 6\%$ (mean $\pm$S.D., $n=20$).

The procedure was applied to several samples suspected to contain residues of NT. For samples containing residues at levels above 2 μg/kg the results are qualitatively in perfect agreement with those obtained by Dutch regulatory methods using HPLC purification and high-resolution GC–MS of methoxim-trimethylsilyl (MOX-TMS) derivatives. In general, however, these latter methods are not applicable at levels below 2 μg/kg, so no comparison at very low levels was possible. Fig. 4 shows the response for a sample containing 1.5 μg/kg NT. Samples containing NT at levels below 2 μg/kg were also analysed by HPLC–RIA [15] using a different antiserum to that used for preparing the MAIC matrix. Both positive and negative results were in good agreement ($n=7$). Under routine conditions approximately ten samples, including the negative and positive controls, can be analysed in one day per technician, not including the GC–MS analysis and data handling.

The total analysis time is dependent on the instrumentation available (automation). With regard to MT the analysis procedure yields very similar results. The simultaneous determination of NT and MT is currently under investigation.

DISCUSSION

In forensic residue analysis it is always necessary to compromise between speed of analysis, sample throughput, cost of analysis and the degree of reliability needed [6]. The number of samples that can be prepared for mass spectrometric analysis, generally regarded as the most powerful technique for residue analyses that demand a high degree of reliability, is usually limited, especially when a relatively difficult matrix such as muscle is to be investigated. One way of compensating partly for this is to develop multi-residue analyses. Ideally the different analytes should remain in the same fraction during the entire procedure. To achieve this it is necessary to control the separation mechanisms during the sample preparation steps. By isolating a series of specific IgGs and combining them into a cocktail it is possible to prepare a medium suitable for isolating a selected group of analytes from an extract. When combined with an effective primary extraction procedure and proper defatting, IAC results in an isolate that allows the detection and identification of picogram amounts of the analytes. An additional advantage of the selective IAC clean-up from a forensic point of view is its contribution to the total reliability of the method. By selecting an antibody with known specificity the possible interference of a number of, e.g., isomeric steroids can be excluded. The method described can therefore be regarded as highly reliable at the sub-μg/kg level for the detection of residues of NT. So far no indication of false-positive results has been obtained. It is believed that the procedure can be extended to other anabolic steroids without affecting the time necessary to perform the analysis. However, a possible limitation is the capacity of the MIAC matrix. Increasing the number of individual IgGs decreases the resulting capacity. Fortunately, for a method to be applied at the low μg/kg level with small samples only very low capacities are needed. Moreover, it was calculated that from the IgG fraction isolated only 1–2% is specific IgG antibody for NT or MT. Further purification, e.g., by affinity chromatography, can therefore significantly increase the capacity. For qualitative purposes the method has one critical reagent, the antiserum. For quantitative purposes, a deuterated internal standard is additionally necessary. Antisera to anabolic agents have been prepared in many laboratories, including ours [17] and some are commercially available.

It can be concluded that coupling an antibody to a chromatographic carrier matrix does not significantly influence the binding capacity. In addition, capacities for IAC matrices are additive. No significant differences were observed between batches of mixed gels each coupled with one type of IgG and batches prepared by mixing the IgGs prior to the coupling. This result is of importance for the preparation of high-performance (silica-based) MIAC matrices which can only be prepared by coupling to a mixture of IgGs. Such a system is currently under development in our laboratory.

Each IAC column used so far can be regenerated and used for an extended period of time, provided that the samples have been properly defatted prior to application. In our laboratory we have used columns more than 25 times over a period of more than one year without a significant decrease in capacity. However, with the use of monoclonal antibodies [16] the problem of restricted availability

no longer exists and single-use columns should be achievable. Based on our experience with the method described we expect that for forensic residue analysis at levels below 1 μg/kg the approach using MIAC after enzymatic digestion of the sample, combined with GC–MS detection, will prove to be economic, very reliable and practically achievable for most of the laboratories involved.

The small size of the test sample (1 g) is also potentially suitable for automated sample clean-up. Such a small test sample, however, requires properly homogenized laboratory samples to be used for analysis and contra-expertise in the forensic control procedure.

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REFERENCES

1 H.H.D. Meyer and S. Hoffmann, Food Addit. Contam., 4 (1987) 149.
2 C.H. van Peteghem, J. Chromatogr., 369 (1986) 253.
3 C.H. van Peteghem and G.M. van Haver, Anal. Chim. Acta, 182 (1986) 293.
4 R.M. Facino, M. Carini, A. Da Forno, P. Traldi and G. Pomps, Biomed. Environ. Mass Spectrom., 13 (1986) 121.
5 J.S. Gaskell and G. Brownsey, Clin. Chem. (Winston-Salem), 29 (1983) 677.
6 C. van de Water and N. Haagsma, J. Chromatogr., 411 (1987) 415.
7 L.A. van Ginkel, H. van Blitterswijk, P.W. Zoontjes, D. van den Bosch and R.W. Stephany, J. Chromatogr., 445 (1988) 385.
8 G. de Groot and K.L. Wubs, J. Anal. Toxicol., 11 (1987) 175.
9 J. Cordonnier, M. van den Heede and A. Heyndrick, Int. Analyst, 1 (1987) 28.
10 R.H. van den Berg, E.H.J.M. Jansen, G. Zomer, C. Enkelaar-Willimsen, C.A. Laan, P.W. Zoontjes, J.J. van de Siepkamp and R.W. Stephany, Preparation and Characterization of Lyophilized Antisera Against 19-Nortestosterone, RIVM Report No. 368303 003, RIVM, Bilthoven, September, 1985.
11 R.H. van den Berg, E.H.J.M. Jansen, G. Zomer, C. Enkelaar-Willemsen, C.A. Laan, P.W. Zoontjes, J.J. van de Siepkamp and R.W. Stephany, Preparation and Characterization of Lyophilized Antisera Against 17α-Methyltestorsterone, RIVM Report No. 368303 004, RIVM, Bilthoven, September, 1985.
12 E.H.J.M. Jansen, R.H. van den Berg, R. Both-Miedema, C. Enkelaar-Willemsen, G. Zomer and R.W. Stephany, in J. Schölmerich, R. Andreesen, A. Kapp, M. Ernst and W.G.J. Wood (Editors), Bioluminescence and Chemiluminescence, New Perspectives, Wiley, Chichester, 1987, pp. 265–268.
13 O.H. Lowry, M.J. Rosebrough, A.L. Farr and R.J. Rarolall, J. Biol. Chem., 193 (1951) 265.
14 D. Rodbard and J.E. Lewald, Acta Endocrinol., Suppl., 147 (1970) 79.
15 E.H.J.M. Jansen, R.H. van den Berg, G. Zomer and R.W. Stephany, J. Clin. Chem. Clin. Biochem., 23 (1985) 145.
16 R.W. Stephany, in Deutsche Veterinärmedizinische Gesellschaft/Arbeitsgebiet Lebensmittelhygiene, Der Tierarzt im Dienst der Lebensmittelhygiene, Garmisch-Partenkirchen, Sept. 29–Oct. 2, 1987, DVG, Giessen/Lahn, 1988, pp. 221–227.
17 R.W. Stephany, CEC Document No. VI/2372/87, European Community, Brussels, 1987.