Journal of Chromatography, 489 (1989) 95–104
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4604

EFFECTIVE MONITORING OF RESIDUES OF NORTESTOSTERONE AND ITS MAJOR METABOLITE IN BOVINE URINE AND BILE

L.A. VAN GINKEL*, R.W. STEPHANY, H.J. VAN ROSSUM, H. VAN BLITTERSWIJK, P.W. ZOONTJES, R.C.M. HOOIJSCHUUR and J. ZUYDENDORP

National Institute of Public Health and Environmental Protection, Laboratory for Residue Analysis, P.O. Box 1, 3720 BA Bilthoven (The Netherlands)

SUMMARY

The results of a newly developed method for the detection and identification of residues of nortestosterone (NT) and one of its major metabolites, 17α-nortestosterone (epiNT) are described. The method is based on sample clean-up by immunoaffinity chromatography and detection by high-performance liquid chromatography and/or gas chromatography–mass spectrometry (selected-ion monitoring). All samples of bile from calves that had been treated with NT contained significant amounts of epiNT (6–18 μg/l). The NT content of these samples, if detectable, was below 1 μg/l. Urine contained, with one exception, less than 1 μg/l epiNT. NT itself if detectable, was, present in urine or bile at levels below 0.1 μg/l. The results corresponds well with results obtained with a radioimmunoassay procedure.

INTRODUCTION

In large parts of Western Europe, one of the most frequently used illegal, injectable anabolic agents for fattening veal calves and cattle is the androgenic steroid 17β-nortestosterone (NT). Owing to extensive metabolism, the NT content of urine and bile from treated animals is very low, typically less than 1 and 10 μg/l, respectively. One of the major metabolites of NT is its 17α-epimer (epiNT). Screening of samples from treated animals for the presence of epiNT with a combined high-performance liquid chromatographic–radioimmunoassay procedure (HPLC–RIA) resulted in virtually no false negative results.

The aim of this investigation was to develop a method for the analytical confirmation of these results based on sample clean-up by immunoaffinity chromatography (IAC), a technique previously applied successfully in residue analysis [1–5]. For this purpose an immunogen [NT-17-hemisuccinate–bovine serum albumin (NT-17HS–BSA)] was synthesized and two rabbits were immunized. The

0378-4347/89/$03.50

resulting antisera showed sufficient affinity for both NT and epiNT. Based on IAC matrices obtained with this antiserum, an assay system was developed that allows the detection of NT and epiNT by HPLC with UV absorbance detection or detection and identification by gas chromatography–mass-selective detection (GC–MSD). In the latter instance quantification is possible using an isotope-labelled internal standard.

EXPERIMENTAL

Chemicals

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

Standards

The standards of NT and epiNT were a gift from Organon (Oss, The Netherlands). All other steroid standards were from Sigma (St. Louis, MO, U.S.A.). All standards were checked for purity by mass spectrometry (MS), Fourier transform infrared spectroscopy (FTIR) and HPLC with UV spectrum detection. The internal standard, [16,16,17α-$^2H_3$] nortestosterone (NT-d3), was synthesized in our Institute as described [2].

Methods

HPLC–RIA for NT and epiNT was performed as described [6], with antiserum by courtesy of Dr. H.H.D. Meyer [7]. The immunogen used for preparing the IAC matrices was prepared from commercially available NT-17-HS (Steraloids, Amsterdam, the Netherlands) and BSA (Sigma) using a carbodiimide coupling procedure. The antiserum used to prepare the immunoaffinity matrix was obtained by immunizing a rabbit four times over a period of five months, each time with 2 mg of the immunogen. The antiserum raised was characterized with a RIA procedure with [6,7-^{3}H]NT (TRQ 2510) (Amersham, Houten, The Netherlands). The data were subjected to Scatchard analyses in order to calculate the affinity constants and bindig capacities [8]. The isolation of the immunoglobulin G (IgG) fraction and coupling to the activated matrix (Tresyl-activated Sepharose®; Pharmacia, Uppsala, Sweden) were performed as described previously [1].

After coupling of an aliquot of the IgG fraction, the binding capacity (ng/ml of gel) was determined using an aqueous solution of NT (5 ng/ml) and [6,7-^{3}H]NT [200 cpm (10 Bq)/ml]. A volume of 15 ml was applied to a column and the eluate was fractionated (1-ml fractions). As soon as the total amount of NT applied exceeded the capacity of the column, the ^{3}H activity was detected in the eluate (LS300 liquid scintillation counter; Beckmann, Fullerton, CA, U.S.A.).

---

[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.

HPLC system

The HPLC system consisted of two solvent delivery systems (Model 510; Waters Assoc., Millipore Division, Milford, MA, U.S.A.), two six-way switching valves and a Spectroflow 773 UV absorbance detector (Kratos, Ramsey, NJ, U.S.A.).

The samples were concentrated on a Hypersil ODS (Shandon, Runcorn, U.K.) column (2 mm×4.6 mm I.D.) using methanol–water (20:80, v/v) as the eluent and subsequently analysed on a Hypersil ODS column (150 mm×4.6 mm I.D.) using methanol–water (65:35, v/v) as the eluent. The flow-rate was 2.0 ml/min and the UV absorbance was detected with a Model 440 detector (Waters Assoc.) at 254 nm. The NT and epiNT fractions were collected with a Redirac 2112 fraction collector (LKB, Bromma, Sweden).

GC–MSD system

The GC–MSD system (Model 5970 mass-selective detector combined with a Model 5890 gas chromatograph; Hewlett-Packard, Avondale, PA, U.S.A.) was used in the selected-ion mode, using a dwell time of 100 ms. The gas chromatograph was equipped with a CpSil 19CB capillary column (Chrompack, Middelburg, The Netherlands) (25 m×0.25 mm I.D.) using helium as the carrier gas at a column head pressure of 70 kPa. The oven was kept at 100°C for 3 min, then the temperature was raised to 280°C at 20°C/min.

General assay protocol

To 5 ml of urine or 1 ml of bile an internal standard solution containing 5 ng of NT-d3 in 0.05 ml of ethanol was added. The pH of the sample was adjusted to 5.2 with acetic acid. To this mixture 1 ml of 1 mol/l acetate buffer (pH 5.2), containing 10 000 Fischman units of β-glucuronidase and 100 000 Roy units of sulphatase (Suc d'*Helix pomatia*: Industrie Biologique Francaise, Villeneuve la Garenne, France), was added, then the sample was incubated for 2 h at 37°C. If necessary, the samples were centrifuged and, after cooling to room temperature, they were applied to the IAC columns which had previously been flushed with water. After washing the columns with 5 ml of water they were eluted with 5 ml of ethanol–water (40:60, v/v). After eluting the columns they were washed with 5 ml of ethanol–water (80:20, v/v), 5 ml of water and 5 ml of 0.1 mol/l phosphate buffer containing 0.05% (w/v) thiomersal and stored at 4°C. The volume of the NT fraction was reduced under a stream of nitrogen in a water-bath at 40°C. Some water was added to make sure that the ethanol content was less than 10% (v/v). The fraction was injected quantitatively onto the described HPLC column-switching system and the NT and epiNT fractions were collected combined. After evaporating the solvent, the dry residue was subjected to derivatization with 0.025 ml of heptafluorobutyric anhydride (HFBA) (Chrompack) in acetone (1:4, v/v). Under these conditions only NT-diHFB is formed in detectable amounts. After evaporation of the solvent the residue was dissolved in 0.025 ml of isooctane. For the GC–MSD analysis 5 μl were injected into the GC–MSD system. Prior to the injection a number of standard solutions were analysed, each con-

taining a fixed amount of the internal standard and various amounts of, e.g., NT. Each standard solution was analysed in duplicate and the responses (A) at the masses (m/z) 669, 666, 453, 306 and 239 were recorded. The intensity ratio A_{669}/A_{666} was used to construct the isotope-dilution (ID) calibration graph whereas the intensity ratios A_{453}/A_{666} and A_{239}/A_{666} of the corresponding diagnostic ions were each averaged and used as identification criteria. The GC–MSD 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_{669}) should be not more 1.2 or less than 0.4 times the average value for A_{669} in the standard solutions. The ratios A_{453}/A_{666} and A_{239}/A_{666} 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_{239}, A_{453}, A_{666} or A_{669} only if the response maximum is clearly higher than the background.

RESULTS

HPLC–RIA screening of urine samples

Fig. 1 shows the results obtained by HPLC–RIA for NT and epiNT for urine samples ($n=66$) from animals in which an NT-containing application site was observed at slaughter. This figure clearly demonstrates that NT, if detectable, is present at levels below 2.5 μg/l whereas epiNT concentrations are much higher, ranging from 0.2 to 19 μg/l.

Characteristics of IAC matrices

Table I gives the major characteristics of the antiserum, obtained after immunizing a rabbit as described under *Methods*, with regard to the binding capacity

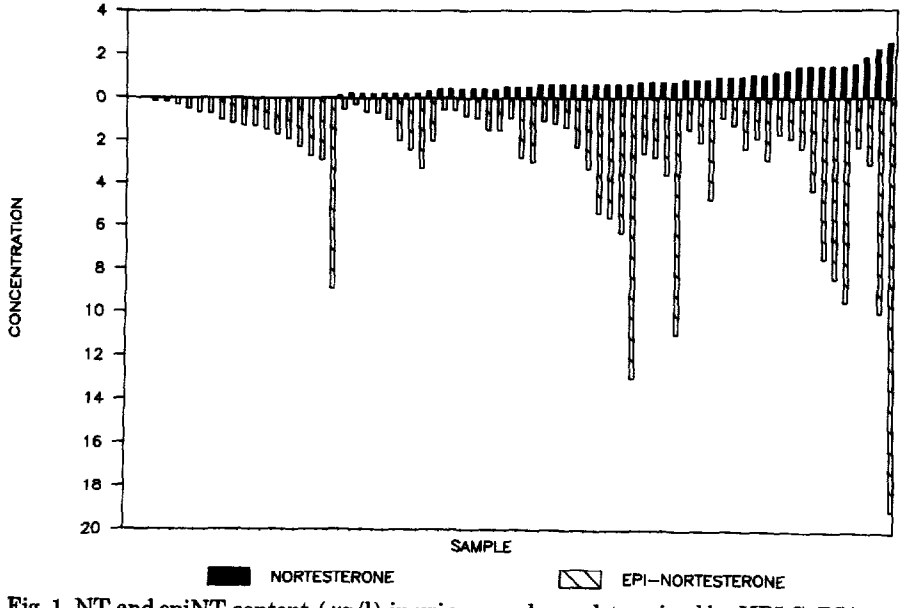

Fig. 1. NT and epiNT content (μg/l) in urine samples as determined by HPLC-RIA.

TABLE I

CHARACTERIZATION OF THE ANTISERUM USED

Serum: anti-NT-17-HS-BSA; batch code 87M1532.

Analyte	K_a (l/mol)	Binding capacity, theoretical (ng/ml)
NT	$2.9 \cdot 10^9$	370
epiNT	$0.8 \cdot 10^9$	790
NT-d3	$4.6 \cdot 10^9$	320

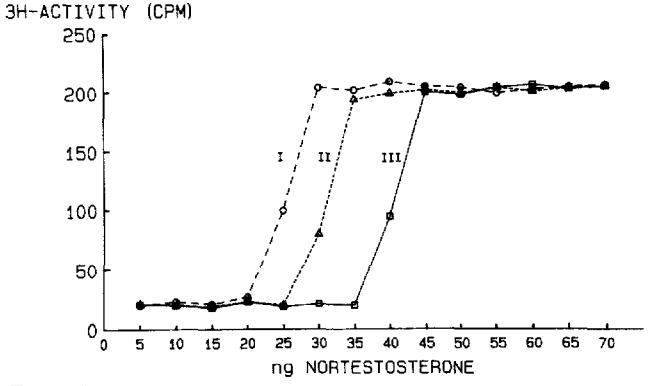

Fig. 2. Determination of the capacity of the IAC matrices for NT. The amount of protein used for preparing the different IAC matrices was (I) 0.31, (II) 0.63 and (III) 1.25 mg/ml of gel.

for NT, its metabolite epiNT and NT-d3. The affinity constants for NT and NT-d3 are not statistically significantly different. The affinity constant for epiNT is slightly lower. The maximum capacity, however, is approximately twice as high for epiNT.

Fig. 2 shows the results of the experiment in which the capacity for NT was determined. It can be concluded that the capacity of the IAC columns ranges from approximately 25 to 40 ng/ml of gel, depending on the amount of IgG used for coupling. Similar graphs were constructed for epiNT. However, the slope of the mid-section was less steep for this compound, probably owing to a lower affinity constant for the [6,7-^{3}H]NT used for detecting non-retained NT. The results are summarized in Table II. In addition the theoretical capacities, based on the results obtained in the Scatchard analysis and the coupling efficiency of the IgG to the activated matrix (ranging from 70 to 94%), are given. The experimental values for NT are roughly twice as high as the calculated values, whereas the corresponding values for epiNT are in close agreement. However, in view of the general assumptions made in the Scatchard analyses and the different testing conditions, free in solution versus covalently coupled to a chromatographic matrix, it can be

TABLE II

BINDING CAPACITIES AS A FUNCTION OF THE AMOUNT OF IgG COUPLED TO THE IAC MATRIX FOR NT AND epiNT

Theoretical values as calculated on the basis of Scatchard analysis and experimental values determined as described under *Methods*.

IgG (mg/ml of gel)	Binding capacity (ng/ml of gel)			
	Theoretical		Experimental	
	NT	epiNT	NT	epiNT
0.31	6	13	20	15
0.63	11	24	25	35
1.25	18	38	35	40

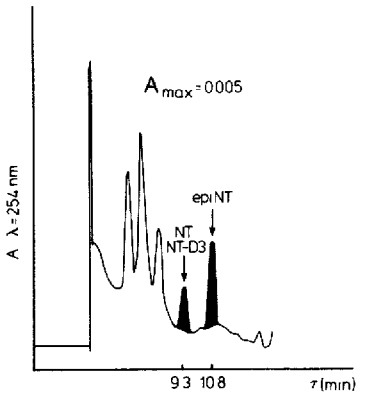

Fig. 3. Chromatogram of a sample of urine obtained from a calf treated with NT.

concluded that the IAC matrix obtained has a capacity that is in agreement with the antiserum used. The matrix did not show any affinity for testosterone or methyltestosterone.

Analysis of urine and bile

Fig. 3 shows an HPLC trace obtained with a sample of urine. NT and epiNT are well separated and no interfering components elute at similar retention times. As we added NT-d3 to all the samples tested, HPLC cannot be used to detect NT in a sample because NT and NT-d3 coelute. For purposes of quantification GC–MSD detection was always used. Fig. 4 shows the mass spectra for NT-diHFB (MW 666) and the internal standard NT-d3-diHFB (MW 669). For NT and epiNT the masses 453 ($C_{22}H_{24}O_2F_7$) and 239 ($C_{18}H_{23}$) correspond to the loss of one and two HFB groups, respectively. Each time the corresponding mass of NT-d3 is 3 units higher. Therefore, these masses can be used as an additional identification criterion. However, mass 306 ($C_{11}H_9O_2F_7$), corresponding to a fragment containing one HFB group, the A ring of the steroid and one methyl group,

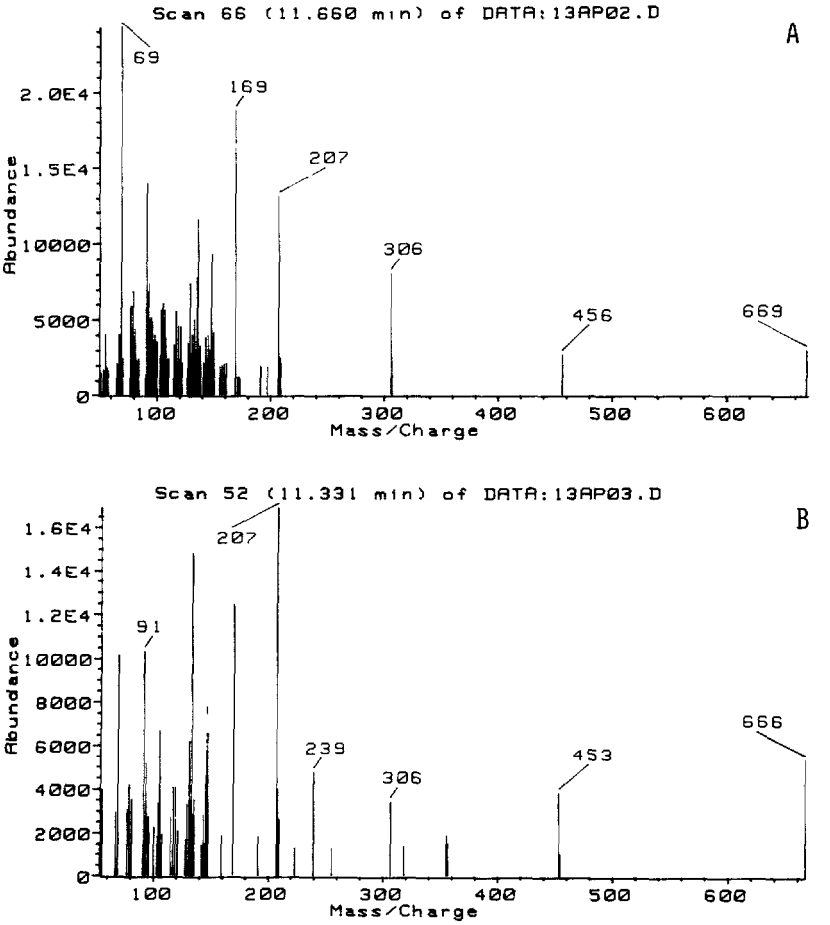

Fig. 4. Mass spectra of NT-diHFB (B) and NT-d3-diHFB (A).

no longer contains the D ring on which the ^{2}H atoms are located. Therefore, it will always be present at the retention time of NT. Fig. 5 shows the mass chromatograms for four of the five ions monitored, obtained for a sample of bile. For this sample it was possible to fulfil all the identification criteria mentioned. In order to confirm the above results, which were obtained by HPLC–RIA, a total of ten samples of urine together with the corresponding samples of bile were analysed using the described procedure. ID–GC–MS calibration graphs were prepared by analysing blank samples after the addition of NT-d3 and various amounts of epiNT and NT. The ID calibration graphs were parallel with those obtained by the direct analyses of standard solutions.

In urine NT, if detectable, was, present in amounts less than 0.1 μg/l. EpiNT concentrations ranged from less than 0.5 μg/l to 3 μg/l ($n=9$). In one sample no epiNT was detected. For bile the situation was different. In all samples NT was detectable, with concentrations ranging from less than 0.5 to 1.0 μg/l. EpiNT was present at concentrations ranging from 7 to 18 μg/l. The correlation between the

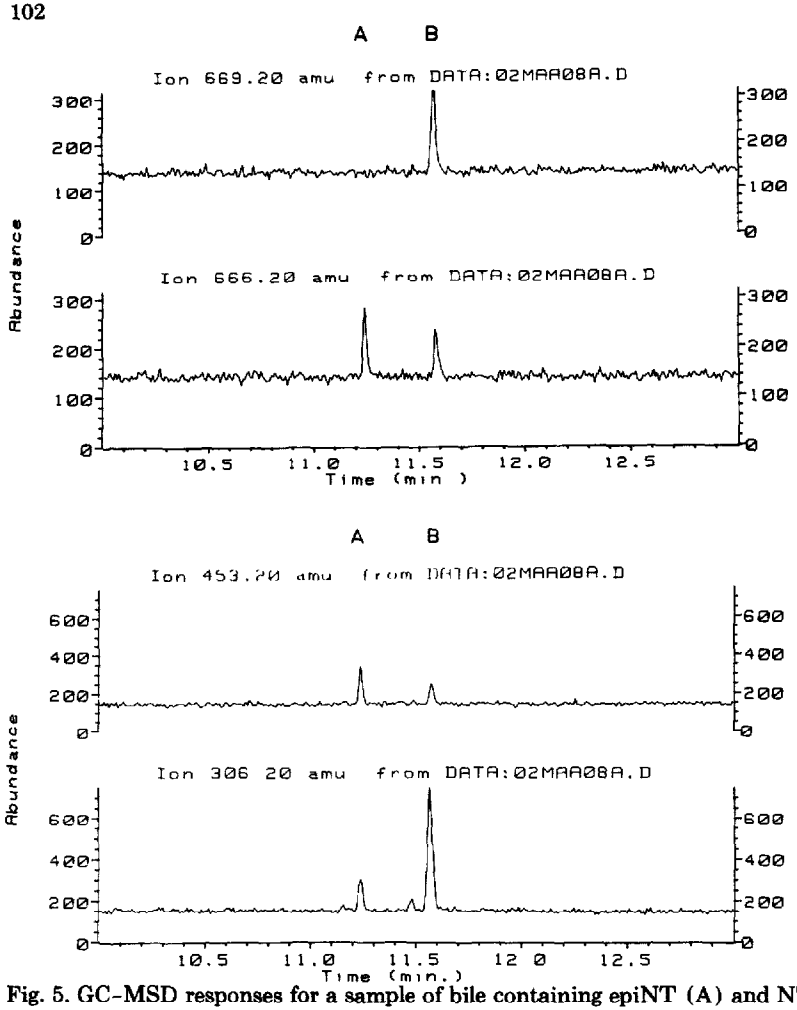

Fig. 5. GC–MSD responses for a sample of bile containing epiNT (A) and NT (B) obtained after derivatization.

epiNT concentrations in urine and bile was significant at $P=0.05$ ($r=0.61$). The recovery of the internal standard was $85\pm6\%$ (mean $\pm$S.D., $n=20$) and not significantly different for urine or bile at 1 and 5 μg/l, respectively.

DISCUSSION

Several methods for screening samples for the presence of residues of anabolics are available. However, for forensic purposes there always remains a need for methods that can confirm results obtained in such a screening procedure.

With the method described it is possible to detect and determine amounts of NT and epiNT below 1 μg/l in urine and bile. For epiNT, HPLC quantification is possible from approximately 1 μg/l; GC–MSD confirmation in this instance is optional. The limit of detection with GC–MSD is 0.1 μg/l for urine and 0.5 μg/l for bile. However, the limit of identification is approximately two or three times higher, because in order to identify NT or epiNT positively all ions monitored

must be present and not only the relatively intense molecular ion. On the other hand, the HPLC step is not necessary in order to obtain an extract suitable for GC–MSD analysis. The limit of detection is only slightly higher for GC–MSD analysis directly following the IAC purification, depending on the criteria for identification used. Therefore, the choice which procedure is to be used (IAC–HPLC, IAC–GC–MSD or IAC–HPLC–GC–MSD) can be made from practical considerations, e.g., sample throughput, cost and purpose of analysis.

The results obtained confirm those of immunochemical analyses, namely that epiNT is a more suitable analyte than NT itself for forensic purposes and, when available, bile is a more suitable matrix than urine. Moreover, the possibilities of IAC combined with relatively simple GC–MSD analysis were clearly demonstrated.

With regard to the technique used for sample clean-up, it can be concluded that it is very useful when the detection and determination of small amounts of analytes are necessary. The matrix described here is different from those described by us elsewhere [2,3]. Here we have the situation in which a single antibody has affinity for two analytes, NT and epiNT. As we use NT-d3 as an internal standard for the quantification of epiNT, special attention must be focused on the question of whether the amount of epiNT in the sample influences the recovery of the internal standard. At present we do have some indications that the behaviours of NT-d3 and epiNT on the IAC matrix are not completely independent.

The columns described have been used many times. For the analysis of urine samples there is no indication that the column performance deteriorates after several runs. For these and other columns [1] there are indications that at least 100 samples per column can be analysed, provided that the samples are centrifuged prior to application and the columns are handled and stored properly. Based on 100 samples per column and a coupling concentration of IgG of 0.5 g/ml of gel, we can perform 3600 analyses with each millilitre of antiserum. When the same antiserum is used in an RIA it is possible to analyse approximately 20 000 samples. This indicates that IAC is mainly suitable for purposes of confirmation of results obtained in a screening procedure. However, when a monoclonal antibody [5] is used, the problem of the availability of a sufficient supply of antiserum no longer exists.

It is our belief that methods based on the detection and identification of analytes by GC–MSD after sample clean-up by IAC are very useful for the determination of low levels of residues of anabolics in biological samples.

ACKNOWLEDGEMENTS

This work was performed within Project 388701 on behalf and for the account of the Dutch Veterinary Chief Inspectorate of Public Health. The antiserum for screening samples by HPLC–RIA for the presence of epiNT was a gift from Dr. H.H.D. Meyer, Institut für Physiologie der Südd. Versuchs- und Forschungsanstalt für Milchwirtschaft der Technischen Universität München. The preparation of the antiserum used for the immunoaffinity chromatograph was performed within our Institute (Animal Supply, J.J. van de Siepkamp) and the standards

were checked for purity by Fourier transform infrared spectroscopy (Laboratory for Analytical–Organic Chemistry, T. Visser).

REFERENCES

1 L.A. van Ginkel, H. van Blitterswijk, P.W. Zoontjes, D. van den Bosch, and R.W. Stephany, J. Chromatogr., 445 (1988) 385.
2 L.A. van Ginkel, R.W. Stephany, H.J. van Rossum, H.M. Steinbuch, G. Zomer, E. van de Heeft and A.P.J.M. Jong, J. Chromatogr., 489 (1989) 111.
3 L.A. van Ginkel, H.M. Steinbuch, H.J. van Rossum, H. van Blitterswijk, P.W. Zoontjes, E. van den Heeft,, A.J.P.M. Jong, and R.W. Stephany, Pharm. Weekbl., Sci. Ed., 10 (1988) 65.
4 W. Haasnoot, R. Schilt, A.R.M. Hamers, F.A. Huf, A. Farjam, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 489 (1989) 157.
5 C. van de Water and N. Haagsma, J. Chromatogr., 411 (1987) 415.
6 E.H.J.M. Jansen, R.H. van den Berg, G. Zomer and R.W. Stephany, J. Clin. Chem. Clin. Biochem., 23 (1985) 145.
7 M. Rapp and H.H.D. Meyer, Arch. Lebensmittelhyg., 38 (1987) 35.
8 D. Rodbard and J.E. Lewald, Acta Endocrinol., Suppl., 147 (1970) 79.