Journal of Chromatography, 489 (1989) 225–234
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4599

MULTI-RESIDUE SCREENING OF BOVINE URINE ON XENOBIOTIC OESTROGENS WITH AN OESTROGEN RADIORECEPTOR ASSAY

C.J.M. ARTS* and H. VAN DEN BERG

TNO-CIVO Toxicology and Nutrition Institute, Department of Clinical Biochemistry, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

SUMMARY

An improved radioreceptor assay (RRA) is used for the screening of urine samples from cattle for the presence of exogenous oestrogenic anabolic compounds, e.g., stilbenes and zeranol. The method includes extraction of the hormones from urine samples with simultaneous purification using reversed-phase C_{18} cartridges. High-performance liquid chromatography is used to isolate the anabolics from the naturally occurring oestrogens. Fractions containing the stilbenes and zeranol are collected and subsequently analysed using the RRA with the oestrogen receptor, isolated from immature calf uteri, as a binder and tritiated 17β-oestradiol as a tracer. Urine samples from untreated calves and cows and samples from calves treated with zeranol–trenbolon acetate, dienoestrol or hexoestrol or samples containing diethylstilboestrol were analysed with this RRA method. Sensitivity, specificity and predictive values were calculated at different classification levels (0.4, 0.5, 0.6 and 1.0 ng/ml 'apparent' oestradiol in urine). An optimum sensitivity (89%) with a maximum specificity (95%) was reached at a classification level of 0.6 ng/ml. At this level the detection limits in urine samples are 0.5 ng/ml for hexoestrol, 0.6 ng/ml for diethylstilboestrol, 0.9 ng/ml for dienoestrol and 5.0 ng/ml for zeranol.

INTRODUCTION

Xenobiotic anabolic compounds have been used as growth promoters in cattle. However, in most countries their use is forbidden, because of their proved or alleged toxic and/or carcinogenic properties. Therefore, to comply with official regulations and to guarantee the absence of xenobiotic anabolic residues in meat and meat products, practical and reliable analytical methods are needed.

As anabolics are often used as a mixed preparation of androgenic and oestrogenic compounds or as oestrogens alone, screening for xenobiotic oestrogens in urine seems appropriate.

The radioreceptor assay (RRA) described is specially designed for screening urine from cattle possibly treated with stilbenes and/or zeranol. Other compounds showing receptor binding activity can also be demonstrated.

0378-4347/89/$03.50

German investigators [1,2] applied an RRA based on the method originally described by Korenman [3] to screening for oestrogenic anabolic compounds. However, they did not differentiate between the compounds of interest (exogenous oestrogenic anabolics) and the naturally occurring oestrogens. Grohman et al. [4] introduced high-performance liquid chromatography (HPLC) as a purification and separation step prior to RRA, but did not evaluate the feasibility of the system.

In this paper an improved RRA is described and evaluated for screening urine samples for the presence of exogenous oestrogenic anabolics. The method includes simultaneous extraction and purification of the anabolic compounds with reversed-phase C_{18} cartridges and HPLC to separate the anabolics from naturally occurring oestrogens.

EXPERIMENTAL

Materials

All chemicals used were HPLC or AnalaR grade. Suc d'*Helix pomatia* was supplied by Boehringer Mannheim (Almere, The Netherlands). Sodium molybdate, aprotinine, ovalbumin, 17β- and 17α-oestradiol, ethynyloestradiol, hexoestrol, dienoestrol, diethylstilboestrol (DES), progesterone, medroxyprogesterone, testosterone, methyltestosterone and cortisol were obtained from Sigma (St. Louis, MO, U.S.A.). Bacitracin was purchased from Serva (Heidelberg, F.R.G.). Sodium acetate and reversed-phase C_{18} cartridges were obtained from J.T. Baker (Deventer, The Netherlands).

Zeranol was kindly provided by Dr. C. Wassink (International Minerals and Chemicals, Terre Haute, IN, U.S.A.). Equol was obtained from Professor D.N. Kirk (Steroid Reference Collection, Chemistry Department, Queen Mary College, London, U.K.). Zearalanone, α- and β-zearalenol, zearalenone and taleranol were kindly provided by Dr. R.W. Stephany [Laboratory for Residue Analysis, National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands]. 17β-Trenbolone and 17α-trenbolone were obtained from Roussel-Uclaf (Romainville, France). 16α-Hydroxyoestrone was kindly provided by Professor J.H.H. Thijssen (Department of Endocrinology, Academic Hospital, Utrecht, The Netherlands). Tritiated compounds were obtained from Amersham (Houten, The Netherlands).

Apparatus

An Ultra-Turrax, a Waring blender, a centrifuge (MSE Coolspin 2) and an ultracentrifuge (Beckman) were used for isolation of the oestrogen receptor.

HPLC was performed with Series 10 constant-flow pump (Perkin-Elmer, Norwalk, CT, U.S.A.), a Model ISS-100 autoinjector (Perkin-Elmer), a Model 202 fraction collector (Gilson, Villiers-le-Bel, France) and a Spectroflow 757 UV–VIS variable-wavelength detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). A Knauer stainless-steel column (125 mm$\times$4.6 mm I.D.) was laboratory-packed with LiChrosorb Diol (5 μm) (Merck, Darmstadt, F.R.G.) or with Hypersil ODS (5 μm) (Shandon, Astmoor, U.K.). As the mobile phase for the

LiChrosorb Diol column 5.5% (v/v) isopropyl alcohol in *n*-hexane was used at a flow-rate of 1.5 ml/min, and for the Hypersil ODS column methanol–water (60:40, v/v).

Isolation of the oestrogen receptor

The cytosolic oestrogen receptor isolated from uteri of calves, rabbits, rats and sheep was dissected as originally described by Korenman [3] and Ingerowsky and Stan [5] and more recently by Arts et al. [6].

Reagents and procedures

A general scheme of the method is given in Fig. 1. A detailed description of reagents and procedures was presented elsewhere [6]; an outline is given below.

Sample preparation

Conjugated compounds in 1.0 ml of urine are hydrolysed enzymatically by 4500 Fishman U β-glucuronidase and 45 000 Roy U sulphatase [corresponding to 0.5 ml of Suc d'*Helix pomatia*, diluted 1:10 in acetate buffer (pH 4.5)]. The tubes are incubated for 2 h at 50°C or overnight at 37°C. The unconjugated compounds are extracted from the hydrolysate by reversed-phase C_{18} cartridges. These cartridges first have to be activated with methanol and water. Then the hydrolysate is transferred to the activated C_{18} cartridges and subsequently washed with distilled water followed by 50% methanol. Finally, the hormones are eluted from the cartridge with 2 ml of methanol. The resulting fraction is evaporated to dryness.

The residue is dissolved in 30 μl of isopropyl alcohol by vortexing and 270 μl of *n*-hexane are added. By means of an autoinjector, 275 μl of the sample are injected on to the HPLC column packed with LiChrosorb Diol and eluted with isopropyl alcohol in *n*-hexane (5.5%, v/v). Fractions in which the stilbenes and zeranol have been eluted are collected and evaporated to dryness at 40°C under a gentle stream of nitrogen. This fraction is used for analysis with the RRA.

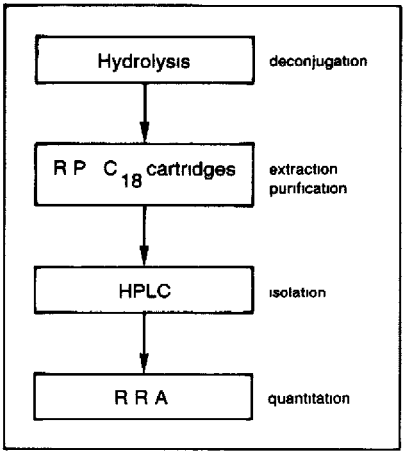

Fig. 1. General scheme of the radioreceptor assay method.

Radioreceptor assay

In this assay 17β-oestradiol is used as a standard (concentration range 0–8000 pg/ml). Tritiated 17β-oestradiol (10 000 dpm; ca. 10 pg in 100 μl of assay buffer is used as a tracer. As a binder 100 μl of the four-fold diluted cytosolic oestrogen receptor isolated from immature calf uteri are used. The tubes are incubated overnight at 4°C. Bound and free fractions are separated by addition of 250 μl of dextran-coated charcoal suspension, incubation at 4°C for 10 min and centrifugation at 1500 *g* and 4°C for 20 min. After addition of 4 ml scintillation liquid to a 500-μl aliquot of supernatant, the radioactivity is measured. The results are calculated with a four-parameter logistic model. Results are expressed as 'apparent' oestradiol in ng/ml in urine.

Samples

To determine the specificity and sensitivity of the method, urine samples from untreated calves and cows and from calves injected with either stilbenes or zeranol were analysed. Urine samples from two calves injected with 100 mg of hexoestrol acetate and samples from two calves injected with 100 mg of dienoestrol acetate were obtained within eighteen days after administration of the anabolics. These animals had been involved in a collaborative experiment by three Dutch governmental institutes: the National Quality Control Institute for Agricultural and Horticultural Products (RIKILT, Wageningen, The Netherlands), the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands) and the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands). The concentrations of hexoestrol and dienoestrol were analysed by a radioimmunoassay (RIA) technique [7]. Urine samples from calves treated with zeranol (36 mg) and trenbolone acetate (140 mg) were obtained within one week after administration of the anabolics by implantation in the base of the ear. This experiment was performed at TNO Cereals, Flour and Bread Institute, ILOB Department (Wageningen, The Netherlands). The concentration of zeranol was analysed by a RIA technique. An anti-zeranol antiserum, raised in sheep, was used as the binder. The cross-reaction of this antibody to zeranol and its metabolites, tested according to Abraham [8], was 100% for zeranol, 48% for taleranol, 34% for zearalenone, 100% for zearalanone, 55% for α-zearalenol and 9.3% for β-zearalenol. Freeze-dried urine samples containing DES or trenbolone were obtained from Dr. R.W. Stephany. These samples were packed in 2-ml portions under dry nitrogen in injection vials. They were aliquots of blended urine pools from control animals and animals treated with anabolics, prepared as quality control samples within Benelux (Anaref) and EC (Bureau Communautaire de Référence, BCR) reference material programmes.

RESULTS AND DISCUSSION

Oestrogen receptor preparation

Oestrogen receptor preparations were isolated from different species, such as calf, sheep, rabbit and rat. Experiments were performed in a search for a quantitatively and qualitatively good receptor preparation. The specific and non-spe-

cific bindings of tritiated oestradiol with increasing volumes of the various receptor homogenates were measured. The highest relative bindings were found with the receptor homogenates from rabbit and calf (Fig. 2). Scatchard analysis [9] indicated similar affinities ($0.5 \cdot 10^{10}$–$1.7 \cdot 10^{10}$ l/mol) for 17β-oestradiol with receptor preparations from calf, sheep, rabbit and rat. From these binding experiments and for practical reasons (receptor yield), we decided to use calf uteri as the source of oestrogen receptor.

In Table I the relative bindings of various compounds to the oestrogen receptor isolated from calf uteri are given. This was assessed by estimating the displacement of the ^{3}H-labelled 17β-oestradiol from the receptor at a relative binding of

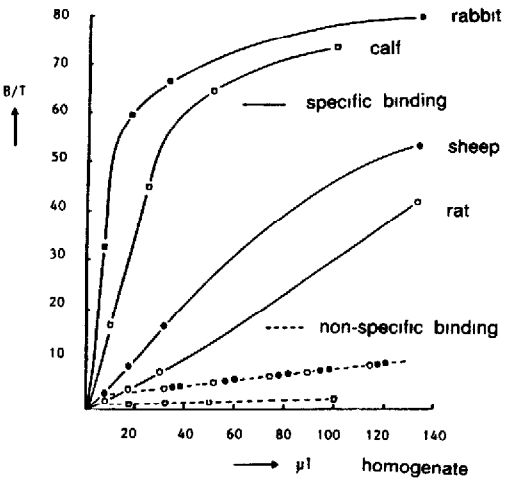

Fig. 2. Specific and non-specific bindings of tritiated 17β-oestradiol to cytosolic oestrogen receptor in homogenates of uteri from different species.

TABLE I

CROSS-REACTIVITIES OF DIFFERENT STEROIDS AND/OR ANABOLIC COMPOUNDS AT A RELATIVE BINDING OF 50% B/B_0

Hormone	Cross-reactivity (%)	Hormone	Cross-reactivity (%)
17β-Oestradiol	100	Zearalanone	0.8
17α-Oestradiol	8	α-Zearalenol	27
Oestrone	8	β-Zearalenol	0.4
Oestriol	14	Equol	0.2
16α-Hydroxyoestrone	15	Progesterone	0.01
Ethynyloestradiol	95	Medroxyprogesterone	0.01
Diethylstilboestrol	73	Testosterone	0.01
Dienoestrol	26	Methyltestosterone	0.01
Hexoestrol	55	19-Nortestosterone	0.01
α-Zearalanol (zeranol)	17	17β-Trenbolone	0.01
β-Zearalanol (taleranol)	3.4	17α-Trenbolone	0.01
Zearalenone	1.4	Cortisol	0.01

50% according to Abraham [8]. 17β-Oestradiol and its metabolites showed significant displacements of the tracer from the receptor, but these compounds are eliminated in the HPLC step. Ethynyloestradiol, DES, dienoestrol, hexoestrol and zeranol and its metabolites all show significant displacement of the oestradiol tracer from the receptor; all of these are collected during HPLC and can be analysed by the RRA.

Isolation of xenobiotics by HPLC

The hormone fraction, extracted from 1 ml urine and partly purified with the C_{18} cartridges, may contain naturally occurring oestrogens such as 17β- and 17α-oestradiol, the stilbenes and zeranol, but also phyto-oestrogens. These plant hormones may be present in urine depending on feed composition [10]. To avoid false-positive results and to improve the sensitivity, these naturally occurring oestrogens had to be excluded. Two alternative approaches, affinity chromatography and HPLC, were compared.

With regard to affinity chromatography, experiments were performed with an anti-oestrogen antiserum linked to Sepharose-4B. This antiserum was raised against 17β-oestradiol–6-carboxymethyloxime–bovine serum albumin and had affinity for 17β- and 17α-oestradiol, oestrone and oestriol. With this step 17β-oestradiol and its metabolites could be eliminated from the urine extract. However, another chromatographic step, such as HPLC, was still needed to eliminate the phyto-oestrogens present in the reversed-phase C_{18} extract. For this reason affinity chromatography was not used. Several HPLC systems in combination with different eluents were thoroughly compared.

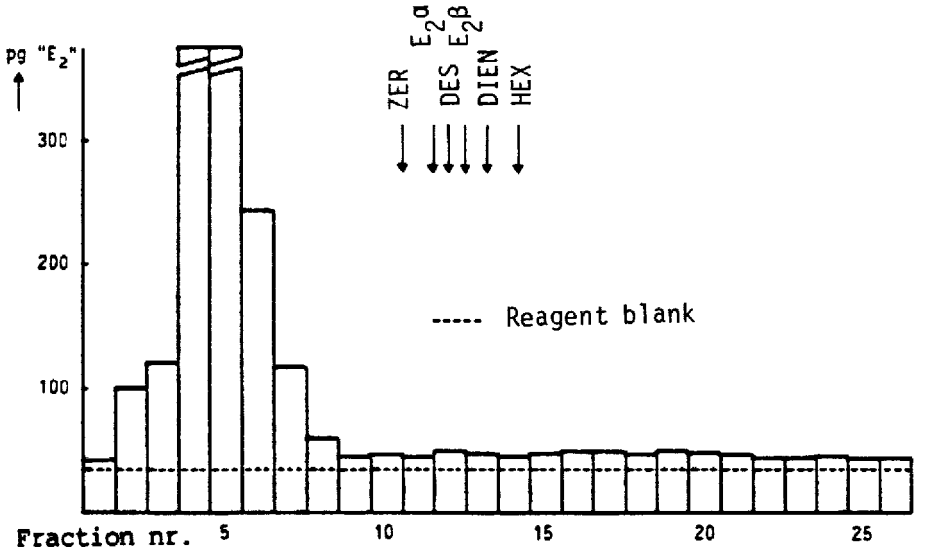

Fig. 3. Elution profile of a urine extract from an untreated calf after HPLC using a 125 mm × 4.6 mm I.D. column filled with Hypersil ODS (5 μm) and methanol–water (60:40, v/v) as mobile phase. Abbreviations: $E_2\beta$ = 17β-oestradiol; $E_2\alpha$ = 17α-oestradiol; HEX = hexoestrol; c-DES = *cis*-diethylstilboestrol; ZER = zeranol; DIEN = dienoestrol; t-DES = *trans*-diethylstilboestrol.

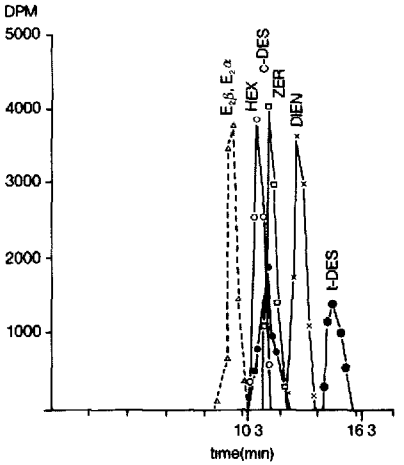

Fig. 4. HPLC profile of labelled naturally occurring oestrogens, stilbenes and zeranol. HPLC was performed using a 125 mm×4.6 mm I.D. column filled with LiChrosorb Diol (5 μm). The mobile phase was 5.5% isopropyl alcohol in *n*-hexane at a flow-rate of 1.5 ml/min. For abbreviations, see Fig. 3.

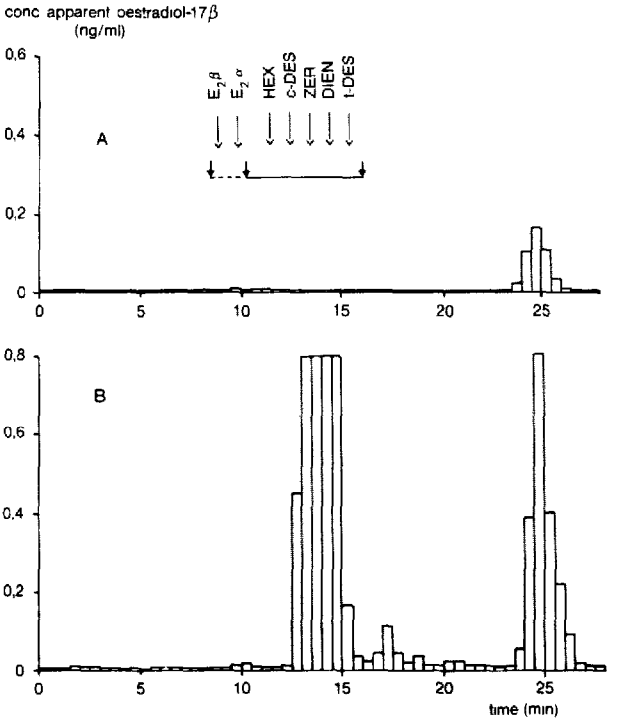

Fig. 5. Elution profiles of a urine extract from (A) an untreated calf and (B) a calf treated with dienoestrol. Fractions every 0.5 min were collected and assayed separately. In (A) the expected 17α-oestradiol/17β-oestradiol fraction is indicated by the broken line and the expected stilbene and zeranol fraction by the solid line. For abbreviations, see Fig. 3.

In Fig. 3 an elution profile of a urine extract is shown, obtained with a Hypersil-ODS column and with methanol–water as the mobile phase. In this system the phyto-oestrogens are first eluted prior to the naturally occurring oestrogens eluting within the stilbene and zeranol fraction. Therefore, in this system affinity chromatography is needed to eliminate oestradiol.

In Fig. 4 an elution profile of an HPLC system is shown, obtained using a LiChrosorb Diol column and isopropanol in *n*-hexane (5.5%, v/v) as the mobile phase. A baseline separation was obtained between 17β- and 17α-oestradiol on the one hand and the stilbenes and zeranol on the other. The phyto-oestrogen fraction appears with a retention time of about 25 min (Fig. 5). This HPLC system was used in the screening method described.

Elution profiles obtained with a urine extract from an untreated calf and from a calf treated with dienoestrol after HPLC are shown in Fig. 5A and B, respectively. In Fig. 5B a stilbene fraction is clearly seen after a retention time of about 15 min. In both elution profiles a receptor-binding fraction is found with a retention time of 25 min. This retention time is identical with that of equol, an important phyto-oestrogen that may be present in urine from cattle. The presence of equol in this fraction was not checked further.

Evaluation of the screening method

For screening purposes the stilbene–zeranol fraction is collected after HPLC, evaporated to dryness and used for analysis with the RRA.

To optimize the classification level, i.e., the concentration at which a sample is positive or negative, the sensitivity and specificity of the method were calculated at different classification levels (Table II). The concepts used were as follows [11]. Sensitivity is defined as the number of true-positives divided by the total number of treated animals tested, equalling the sum of true-positives and false-negatives. For an ideal screening the sensitivity should equal 100%. Specificity is defined as the number of true-negatives divided by the total number of untreated animals screened, the latter equalling the sum of true-negatives and false-positives. For an ideal screening method the specificity should be as high as possible, ideally 100%. The predictive value of a positive test result can be calculated by dividing the number of true-positives by the sum of true-positives and false-pos-

TABLE II

DEFINITIONS AND CALCULATIONS OF SENSITIVITY, SPECIFICITY AND PREDICTIVE VALUES OF TEST RESULTS [11]

Sensitivity: TP/(TP+FN). Specificity: TN/(FP+TN). Predictive value of a positive test: TP/(TP+FP). Predictive value of a negative test: TN/(TN+FN).

	Positive test result	Negative test result
Treated calves	True positive (TP)	False negative (FN)
Untreated calves	False positive (FP)	True negative (TN)

TABLE III

SENSITIVITY, SPECIFICITY AND PREDICTIVE VALUES OF THE RRA METHOD AT DIFFERENT CLASSIFICATION LEVELS BASED ON RESULTS OBTAINED WITH URINE SAMPLES FROM TREATED AND UNTREATED ANIMALS

	Classification level (ng/ml)			
	0.4	0.5	0.6	1.0
Treated ($n=47$):				
Positive (n)	42	42	42	36
Negative (n)	5	5	5	11
Untreated ($n=132$):				
Positive (n)	19	11	7	2
Negative (n)	113	121	125	130
Sensitivity (%)	89	89	89	77
Specificity (%)	86	92	95	98
Predictive value of positive test result (%)	69	79	86	95
Predictive value of negative test result (%)	96	96	96	92

itives. The predictive value of a negative test result can be calculated by dividing the number of true-negatives by the sum of true-negatives and false-negatives.

These parameters were calculated for different classification criteria (Table III). Arbitrarily, 0.4, 0.5, 0.6 and 1 ng/ml were chosen classification criteria or limits of decision, i.e., the concentration of apparent oestradiol at which a urine sample is considered positive or negative.

Up to a limit of decision of 0.6 ng/ml the sensitivity did not change, but at a limit of decision of 1.0 ng/ml 11 out of 47 samples from treated animals proved false-negative, so the sensitivity was 77%. As can be predicted, the specificity is higher when the limit of decision is increased.

On the basis of the sensitivity, specificity and the predictive values for both a positive and a negative test result, we chose for a limit of decision 0.6 ng/ml apparent oestradiol in urine. On the oestradiol calibration graph for the assay this value corresponds to a relative binding of about 32%. At this B/B_0 level the detection limits in urine samples are 0.5 ng/ml for hexoestrol, 0.6 ng/ml for DES, 0.9 ng/ml for dienoestrol and 5 ng/ml for zeranol.

CONCLUSION

The great advantage of this method is that it can be used as a multi-residue method with an analytical sensitivity comparable to that of RIA. A specificity of 95% and a sensitivity of 89% were calculated. Specific antisera, required for the measurement of individual compounds by RIA, are not necessary. With a receptor as binding agent, the receptor-binding affinity of the residues, which reflects the biological activity in vivo, is measured instead of the immunochemical activity, as in RIA. However, a positive test result always has to be analysed further

and confirmed by more specific techniques such as mass spectrometry or HPLC–RIA.

REFERENCES

1 O. Aghte, Arch. Lebensmittelhyg., 27 (1976) 127.
2 P. Rockel, Thesis, Institut für Physiologie der Süddeutschen Versuchs- und Forschungsanstalt für Milchwirtschaft der Technischen Universität München-Weihenstephan, Munich, 1976.
3 S.G. Korenman, J. Clin. Endocrinol., 28 (1968) 127.
4 H.G. Grohman, S. Jordan and H.J. Stan, Fresenius Z. Anal. Chem., 311 (1982) 399.
5 G.H. Ingerowsky and H.J. Stan, Dtsch. Lebensm.-Rundsch., 74 (1978) 1.
6 C.J.M. Arts, P.T.W. Kemperman and H. van den Berg, Food Addit. Contam., (1989) in press.
7 W.G. de Ruig, M.C.J. Berghmans, G.D. Bruchem and Th.H.G. Polman, unpublished results.
8 G. Abraham, in M.K. Schwartz (Editor), Handbook of Radioimmunoassay. Clinical and Biochemical Analysis, Marcel Dekker, New York, 1977, p. 87.
9 G. Scatchard, Ann. N.Y. Acad. Sci., 51 (1949) 660.
10 H.R. Lindner, in F.C. Lu and J. Rendel (Editors), Anabolic Agents in Animal Production, FAO/WHO Symposium, Rome, 1975, pp 151–158.
11 S.R. Gambino, in A. Albertini, R.P. Ekins and R.S. Galen (Editors), Cost/Benefit and Predictive Value of Radioimmunoassay, Elsevier, Amsterdam, 1984, p. 3.