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Potential of receptor–ligand interactions for sample handling in liquid and gas chromatography

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Abstract

The use of receptor proteins for biospecific sample handling in liquid and gas chromatography is described. As a model system the uterine estrogen receptor was chosen for the isolation of 17β-estradiol and its synthetic agonist diethylstilbestrol. Biochemical characteristics relevant for the use of receptors in sample handling such as the kinetics of receptor–ligand binding, reproducibility and capacity are examined by means of an estrogen radioreceptor assay. Different techniques for the non-covalent immobilization of the estrogen receptor were investigated. Both protamine-coated glass fibre filters and silica particles which bind the receptor via electrostatic interactions have been used for this purpose. The use of the estrogen receptor in the isolation of 17β-estradiol and diethylstilbestrol prior to GC–MS analysis is demonstrated and discussed.

1. Introduction

The control on the use of illegal growth promotors in animal production has become an important issue in the European Community in recent years. This resulted in an increased demand for analytical methods which are able to identify and quantitate not only known drugs but also newly synthesized agonists. Sample pretreatment methods based on immunoaffinity interactions have been applied for this purpose; however, they fail in isolating all agonists, which are structurally different. The only common property of these compounds is their biological activity. Moreover, antibody–antigen interactions do not reflect biological activity. On the other hand, binding of ligands to receptors is a measure for biological activity. We therefore investigated the use of receptors in sample handling which due to the group selectivity should allow the isolation of a broader range of compounds.

Currently the application of receptors in the determination of *e.g.* hormonal anabolic compounds such as 17β-estradiol (17βES) and diethylstilbestrol (DES) is restricted to radioreceptor assays (RRAs) mainly [1]. Following C_{18} preconcentration, reversed-phase LC separation and fraction collection, the amount of estrogenic anabolic compounds is determined using an estrogen RRA. This method is used for routine screening of urine samples of cattle for the presence of growth promotors prior to GC–MS determination. While the RRA is a multi-residue method with high sensitivity it is rather laborious and requires long incubation times.

The use of receptors in sample handling techniques has been described by Banner *et al.* [2] for the determination of progesterone and several of its metabolites. The sample handling procedure included an incubation step with the

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progesterone receptor ligand-binding domain, expressed as a protein A fusion protein in *Escherichia coli*. The covalent immobilization of the estrogen receptor (ER) was investigated by Ikeda *et al.* [3] to study association and dissociation phenomena. They reported a significant change in kinetics for the immobilized ER compared to the ER in solution.

In this paper we describe the study to the application of receptor–ligand interactions for biospecific sample handling. The ER was used to demonstrate the potential of the use of receptor–ligand interactions in the isolation of 17βES and DES prior to GC–MS determination. Special attention is paid to important biochemical parameters such as binding capacity, kinetics of association and dissociation and receptor stability. Different possibilities to immobilize the ER on silica or glass fibre filters were investigated. Ligands bound to the receptors were recovered by extraction with ethyl acetate at low pH and identified and quantitated by GC–MS.

2. Experimental

2.1. Apparatus

A B. Braun (Melsungen, Germany) Potter S homogenizer, a Waring (New Hartferd, CT, USA) blender, a DuPont (Den Bosch, Netherlands) Sorvall RC-5B centrifuge and a Beckman (Berkeley, CA, USA) L8-50M/E ultracentrifuge were used for the isolation of the ER from calf uterus. A Jouan (Saint Nazaire, France) GR 2000 SX cooled centrifuge was used in the protamine precipitation RRA. An LKB (Turku, Finland) Wallac 1214 RackBeta "Excel" liquid scintillation counter and Packard (Meriden, USA) Pony Vial 6000292 counting vials were used for radioactivity measurements. A Millipore (Bedford, MA, USA) 1225 sampling manifold filtering device equipped with Whatman (Maidstone, UK) glass fibre filters was used in the filter experiments. A HBI Vortex evaporator (Saddle Brook, NJ, USA) was used for the evaporation of ethyl acetate. A Varian (Sunnyvale, CA, USA) 3400 gas chromatograph equipped with a column (30 m × 0.25 mm I.D.) coated with a DB-5 stationary phase (0.20 μm film thickness) was used for separation of the ligands. A Finnigan (Bremen, Germany) MAT 900 mass spectrometer was used for detection.

2.2. Chemicals

All chemicals used were of analytical grade. All aqueous solutions were prepared using water purified with a Millipore Milli-Q system. Sodium molybdate, aprotinine, protamine sulphate, ovalbumin, 17βES, DES, ethylenediaminetetraacetic acid (EDTA) and dithiotreitol were purchased from Sigma (St. Louis, MO, USA). Bacitracin was obtained from Serva (Heidelberg, Germany). Tris(hydromethyl)aminomethane hydrochloride (Tris) was obtained from Aldrich (Steinheim, Germany) and silica 60 (9–10 μm particle size) was obtained from Merck (Darmstadt, Germany). Hydrochloric acid, methanol, isooctane and ethyl acetate were obtained from J.T. Baker (Deventer, Netherlands). N,O,-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was obtained from Pierce (Rockford, IL, USA). Polychlorobiphenyl-138 (PCB-138) was obtained from Schmidt (Amsterdam, Netherlands). Tritium-labelled 17βES ([^{3}H]17βES) was obtained from DuPont (Dreiech, Germany). Emulsifier-SAFE scintillation liquid was obtained from Packard (Meriden, USA). [16,16,17-$^2H_3$]17βES (99.5 atom% ^{2}H) was obtained from MSD (Montreal, Canada). [$^2H_6$]DES (No. 91M3507) was kindly donated by the Laboratory of Residue Analysis (RIVM, Bilthoven, Netherlands).

2.3. Reagents

Dilution buffer consisted of 15 mmol/l dithiotreitol and 15 mmol/l EDTA in 10 mmol/l Tris · HCl buffer of pH 7.5. Homogenization buffer consisted of 0.25 mol/l sucrose, 25 mmol/l sodium molybdate, 0.1 mmol/l bacitracin, aprotinin [80 kallikrein inhibitor units (KIU) per ml] and 10% glycerol in dilution buffer. Assay buffer consisted of dilution buffer containing

0.1% (w/v) ovalbumin. Precipitation buffer consisted of 15 mmol/l dithiotreitol, 1.5 mmol/l EDTA and 0.1% (w/v) protamine sulphate in 10 mmol/l Tris · HCl buffer, pH 8.0. Coating buffer consisted of 15 mmol/l dithiotreitol, 1.5 mmol/l EDTA and 0.3% (w/v) protamine sulphate in 10 mmol/l Tris · HCl buffer, pH 8.0. A 17βES tracer solution contained [2,4,6,7-$^3H_4$]17βES (specific activity 111.6 Ci/mmol) in methanol to a concentration of 0.4 ng/ml. A 17βES standard solution contained 17βES in methanol to a concentration of 1 μg/ml.

2.4. Procedures

Biochemical characterization

Isolation of the ER. The cytosolic ER was isolated from immature calf uteri as described earlier by Ingerowsky and Stan in 1978 [4]. All procedures were performed at 4°C according to the same method used by Arts *et al.* [1]. Freshly dissected uterine tissue was chopped. The tissue was homogenized 1:1 with homogenization buffer in a Waring blender. The total volume was doubled with homogenization buffer and pottered in a Potter S. The homogenate was centrifuged at 1500 *g* and 4°C for 15 min, and the supernatant at 300 000 *g* and 4°C for 50 min subsequently. The supernatant which contained the ER was frozen and stored at −80°C in aliquots of 3.5 ml.

Radioreceptor assay. An estrogen RRA has been developed which is based on the ability of protamine (a cationic peptide with a molecular mass ranging from 5000 to 10 000) to form a precipitate with the ER [5,6]. After incubation (2 h at 20°C) of the receptor with radioligand in a 4-ml counting vial the receptor-bound fraction and unoccupied receptors were precipitated by addition of an equal amount of precipitation buffer, followed by 30 s of vortexing. After centrifugation at 3500 *g* and 4°C for 15 min the supernatant was decanted and the precipitate was washed three times with 3 ml of water cooled on ice. Finally, the receptor–ligand complex was broken by addition of 400 μl of an aqueous sodium hydroxide solution (0.4 mol/l). All assays were performed in triplicate unless stated otherwise. Scintillation liquid was added and radioactivity was counted after vortexing of the vial contents for 30 s. Non-specific binding (NSB) was determined in an identical way, however in the presence of an additional 100-fold excess of non-radioactive 17βES.

Determination of receptor capacity. Aliquots of 400 μl of an eight times diluted receptor solution were incubated during 2 h at 20°C with increasing amounts of [^{3}H]17βES, ranging from 0 to 200 pg. For the determination of the NSB a 100-fold excess of non-radioactive 17βES was added to each vial. The incubates were treated as described in the previous section. The capacity of the receptor solution was derived from saturation plots according to Michaelis–Menten. Day-to-day variation of capacity was examined by performing the saturation experiments on three different days with the same batch of receptor solution.

Kinetics of receptor–ligand association and dissociation. The association rate of receptor–ligand complex formation was examined ($n = 4$) by incubating 6 ml of a ten times diluted receptor solution at 20°C with 250 pg [^{3}H]17βES in a polypropylene tube. At certain time intervals 200-μl samples were taken from each tube to determine the amount of [^{3}H]17βES specifically bound per milligram protein. For the separation of receptor-bound ligand from free ligand, each sample was sucked through a protamine-coated glass fibre filter onto which the receptor–ligand complex was adsorbed [7,8].

The dissociation rate of the receptor–ligand complex was investigated ($n = 3$) by means of comparison of specific binding (SB) and NSB of [^{3}H]17βES to the vials between the described protamine precipitation assay (20 min) and an extended protamine precipitation assay (50 min). Aliquots of 200 μl of a 4-ml incubation mixture consisting of 200 pg of [^{3}H]17βES dissolved in a four times diluted cytosol solution were incubated for 2 h at 20°C to establish total binding. NSB to receptor solution constituents as well as

to the vials was determined as mentioned previously. In the extended assay receptor–ligand–protamine precipitate derived after centrifugation and washing (see section *Radioreceptor assay*) was vortexed with 2 ml of dilution buffer and centrifuged again at 3500 *g* and 4°C for 15 min. The precipitate was then washed for the second time and centrifuged an additional 7 min at 3500 *g* and 4°C. Finally, 400 μl of a 0.4 mol/l sodium hydroxide solution in water were added to dissolve the precipitate and break the receptor–ligand complex. Radioactivity was measured subsequently.

Trapping of the ER after incubation

Glass fibre filters. Protamine-coated glass fibre filters were prepared by soaking the filters in coating buffer for 1 h. The filters were placed in a Millipore filtering device and washed with a total volume of 9 ml of dilution buffer before use. A 4-ml volume of a four times diluted cytosol solution was incubated with 240 pg of [^{3}H]17β-ES. The estrogen receptor–ligand complex was then trapped on the protamine-coated filters. NSB to receptor solution constituents was determined in the same way, but in the presence of a 100-fold excess of non-radioactive 17β-ES. NSB of the radioligand to the filters was examined by applying an aliquot of 200 μl of an incubation mixture consisting of 4 ml assay buffer and 240 pg of [^{3}H]17β-ES onto each of the filters. After application of the samples onto the filters a reduced pressure of 50 mbar was used to suck the sample through the filter which was then washed three times with 3 ml of dilution buffer. Next the filters were dried at a 200 mbar reduced pressure and transferred to counting vials. Finally, 4 ml of scintillation liquid was added and 2 h afterwards radioactivity was counted.

Silica 60. Silica 60 (9–10 μm particle size) was coated with protamine by gently shaking 1 g of silica in 10 ml of coating buffer overnight. After centrifugation at 2000 *g* during 10 min the supernatant was decanted. The pellet was washed twice with 6 ml of dilution buffer and vortexed for 1 min. Different amounts of slurries of both protamine-coated and plain silica were mixed with 200 μl of an incubation mixture consisting of 5 ml of an eight times diluted receptor solution which was previously incubated with 270 pg [^{3}H]17β-ES. After vortexing for 30 s and a 5 min incubation time the mixture was transferred onto a single uncoated GF/C filter which was placed in the filtering device and washed and dried according to the procedure as described for the glass fibre filters.

Application of the ER in sample handling followed by GC–MS

A 4-ml volume of a four times diluted receptor solution was used to isolate amounts of 1 ng and 250 pg, respectively, of 17β-ES, DES and a 1:1 mixture containing both ligands ($n = 3$). Incubation took place for 2 h at 20°C under gentle shaking. Isolation of the receptor–ligand complex from other matrix constituents was performed with the previously described filtering technique using two protamine-coated GF/C filters. The filters were transferred into glass tubes and the ligands were extracted with 5 ml ethyl acetate after breaking of the receptor–ligand complex with 1 ml of a 0.1 mol/l hydrochloric acid solution. Finally, a 4-ml volume of the organic phase was evaporated to dryness in a vacuum Vortex and 500 μl of methanol were added to dissolve the residue, of which an aliquot of 400 μl was evaporated and derivatized with 100 μl of BSTFA–TMCS at 60°C during 1 h. After evaporation of the derivatization mixture, the residue was dissolved in 25 μl isooctane in which 1 ng/μl PCB-138 was dissolved (external standard). Finally, 1 ng of both [$^2H_6$]DES and [$^2H_3$]17β-ES were added as internal standards. Aliquots of 2 μl of the final mixture were injected onto the gas chromatograph at a carrier (helium) gas flow of 25 cm/s, split flow of 20 ml/min, split time of 1 min, injector temperature of 250°C and interface temperature of 260°C. The following temperature programme has been used: 175°C (1 min), 175–200°C (10°C/min), 200–260 (2.5°C/min) and 260°C (14 min). High-resolution electron impact (70 eV) MS detection, as described earlier [9], was carried out in the

positive ion mode, at a source temperature of 225°C. The signal at different m/z ratios was determined by selected ion monitoring (SIM).

3. Results and discussion

3.1. Principle of biospecific sample handling

Receptor–ligand complex is trapped on a protamine-coated surface. The sample handling technique consists of an incubation step in which the sample is allowed to react with the receptor. Subsequently, both receptor and receptor–ligand complex are trapped on a protamine-coated surface. After removal of free ligand and non-binding matrix components the receptor–ligand complex is dissociated at low pH, the analyte extracted with ethyl acetate and determined by GC–MS.

3.2. Biochemical characterization

The receptor concentration, kinetics of association and dissociation of the receptor–ligand complex and temperature dependency of complex formation were determined in order to evaluate their influence on sample capacity, speed of preconcentration and stability of the receptor during sample handling.

Determination of receptor capacity

Mean values for the equilibrium dissociation constant (K_d) of the 17βES–receptor complex and the total concentration of specific binding sites ($[R]_T$) and standard deviations were obtained by means of saturation experiments at three different days. The capacity of the eight times diluted receptor solution yielded 536 ± 80 pmol/l, meaning that 1 ml of undiluted receptor solution contains 4.3 pmol of specific binding sites. The K_d yielded 172 ± 3 pmol/l. From the K_d, which reflects the ligand concentration at which 50% of the total binding sites are occupied, the binding affinity K_a can be derived according to the relation $K_a = 1/K_d$. The average binding affinity of the receptor for 17βES at 20°C yields $5.8 \cdot 10^9$ l/mol. Day-to-day variation of 2% for K_d demonstrates the high reproducibility of receptor–ligand complex formation. In comparison to immobilized antibody supports the capacity using 1 ml receptor solution is approximately 20 times lower. Batch-to-batch variation in K_d and $[R]_T$ can occur. This is mainly determined by the quality of the tissue and by the conditions during isolation (temperature and time needed for different isolation steps).

Kinetics of receptor–ligand association and dissociation

Fig. 1 shows the relation of SB *vs.* incubation time displayed as a typical association curve. From the association curve the association rate constant (k_{+1}) and the dissociation rate constant (k_{-1}) of the complexation reaction of the ligand and the receptor in equilibrium can be calculated [10]. This yields $k_{+1} = 532 \cdot 10^6$ $(\text{mol/l})^{-1}$ min^{-1}.

Association, as can be seen in Fig. 1, is completed within 30 min under the described experimental conditions. This, however, does not have to be a limitation for the application of receptor–ligand interactions in a dynamic system.

Data on the experimental estimation of dissociation of the receptor–ligand complex are shown in Table I. No significant difference in SB between the conventional protamine precipitation assay of 20 min and the extended assay of 50 min is observed, implying that dissociation does not occur within 50 min. NSB of [^{3}H]17βES to

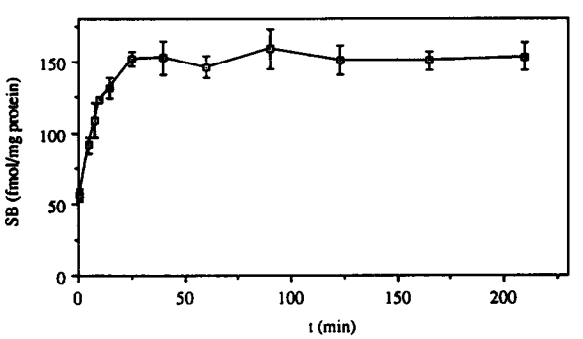

Fig. 1. Graphical representation of specific binding (SB), expressed as fmol [^{3}H]17βES bound per mg protein *vs.* time (t) as a typical association curve ($n = 4$).

Table I
Different values of relative binding (%B/T) and non-specific binding (NSB) of the radioligand to the vials, expressed as percentage of specific binding (SB), for the 20-min protamine precipitation (PP20) assay and for the extended protamine precipitation assay (PP50) of 50 min ($n = 3$)

	%B/T	NSB (%SB)
PP20	80	4.9
PP50	84	1.2

the vials is even lower in the 50 min assay. The k_{-1} can not be determined by substitution of the K_d and the association rate constant in the equation $k_{-1} = K_d k_{+1}$, since both values were determined by different methods.

3.3. Trapping of the ER after incubation

To facilitate the recovery of bound ligand after incubation the estrogen receptor–ligand complex was trapped on a protamine-coated surface. Protamine-coated glass fibre filters or silica were investigated for this purpose. Table II summarizes the properties of several filters with respect to their capability of binding receptor–ligand complex in proportion to the protamine precipitation method. For this purpose the trapping efficiency (%B/T), *i.e.* the percentage of the total amount of [^{3}H]17βES specifically bound to the receptor, was determined for each filter as well as for the protamine precipitation method. It can be concluded that protamine-coated GF/C filters containing two filter layers are most efficiently trapping the receptor–ligand complex. The %B/T equals the value of that of the protamine precipitation method, whereas NSB is even slightly lower.

Attempts to immobilize the ER to protamine-coated filters prior to incubation resulted in recoveries of only 10%. The low recovery can probably be attributed to the low surface density of active binding sites on the filter.

Alternatively to the tested glass fibre filters the trapping properties of protamine-coated silica were investigated. Table III shows the trapping efficiencies of protamine-coated silica and protamine precipitation experiments. Trapping efficiency of protamine-coated silica (P-Si50) and the protamine precipitation method (PP) are approximately the same. Moreover, 50 mg of silica is sufficient for complete recovery of the receptor–ligand complex, since no significant difference in trapping efficiency for the different amounts of silica is observed. The difference in %B/T between coated and plain silica is more clearly than for the filters.

Data shown in Tables II and III exhibit similar

Table II
Different values of relative binding (%B/T) and non-specific binding (NSB) of the radioligand to the vials/filters, expressed as percentage of specific binding (SB) for the different filters

	%B/T	NSB (%SB)
PP	79	5.5
1 × GF/C	20	1.5
2 × GF/C	46	1.5
1 × P-GF/C	45	1.2
2 × P-GF/C	73	1.5
P-GF/B	57	1.3

PP = Protamine precipitation assay; 1 × GF/C = uncoated GF/C filter; 2 × GF/C = two uncoated GF/C filters; 1 × P-GF/C = protamine-coated GF/C filter; 2 × P-GF/C = two protamine-coated GF/C filters; 1 × P-GF/B = protamine-coated GF/B filter ($n = 3$).

Table III
Different values of relative binding (%B/T) and non-specific binding (NSB) of the radioligand to the vials/silica material (NSB), expressed as percentage of specific binding (SB) for the different amounts of silica, either coated or uncoated with protamine

	%B/T	NSB (%SB)
PP	69	7.4
Si50	37	4.5
Si150	36	5.5
P-Si50	65	3.0
P-Si150	59	4.1

PP = Protamine precipitation assay; Si50 = 50 mg of uncoated silica; Si150 = 150 mg of uncoated silica; P-Si50 = 50 mg of protamine-coated silica; P-Si150 = 150 mg of protamine-coated silica ($n = 3$).

trapping properties for both protamine-coated glass fibre filters and silica. In terms of automation silica is preferred to glass fibre filters since silica can be incorporated more easily into conventional solid-phase extraction techniques.

3.4. Application of the ER in sample handling followed by GC–MS

Fig. 2 shows mass chromatograms at different m/z ratios obtained after isolation of a 1:1 mixture of 1 ng of 17βES and DES with the ER solution and subsequent derivatization of the extract. By means of SIM at m/z 412 and 413 the isotope ratio of DES-TMS (peaks 1 and 2, respectively) could be determined. SIM at m/z 416 and 417 yielded the isotope ratio of the 17βES-TMS derivative (peaks 3 and 4, respectively). SIM at m/z 418 shows the derivatized internal standard [$^2H_6$]DES-TMS (peak 5), whereas at m/z 419 the ^{13}C isotope of [$^2H_6$]DES-TMS (peak 6) as well as the derivative of the other internal standard [$^2H_3$]17βES-TMS (peak 7) are represented. Overall recoveries (including derivatization for GC) are 59 and 68% for 1 ng and 250 pg of 17βES, respectively and 5 and 15% for 1 ng and 250 pg of DES, respectively. Incubation of the ER solution with 1 ng of both ligands reveals recoveries of 47 and 7%, respectively. Since an endogenous amount of 17βES is present in the receptor solution (120 pg/ml), recoveries for 17βES are higher than theoretically possible. For the same reason recoveries for DES after isolation of DES only are low, due to competition with endogenous 17βES.

These experiments demonstrate that receptor–ligand interactions can be implemented in sample handling techniques for steroids in the absence of interfering matrix components. In experiments not described calf urine has been pretreated on a XAD-2 precolumn. The column extract was added to a protamine precipitation assay and the amount of receptor–ligand complex was determined and compared to that obtained with a protamine precipitation assay in the absence of the extract. This experiment showed that the presence of the calf urine extract in a binding assay reduced the maximal binding with approximately 60%. Determination of steroids in calf urine pretreated on a XAD-2 precolumn only is hindered by the presence of high concentrations of competing compounds, such as phytohormones and lignanes. The biological activity of these compounds which occur in many forage plants has been described extensively [11–14]. Banner *et al.* [2] also reported an extensive sample pretreatment prior to the isolation of the ligands with the receptor ligand-binding domain, necessary to obtain optimal conditions for receptor–ligand complex formation.

4. Conclusions

The receptor–ligand complex can efficiently be trapped after incubation on either protamine-coated glass fibre filters or silica. The experiments with the protamine-coated filters show that adsorption of the complex to the coated filters occurs within a few seconds. Moreover, washing of the filters after adsorption of the complex, which reduces NSB, did not lead to a decrease in recovery of the steroids investigated. These experiments reveal a strong interaction between the ER and the protamine coating.

It is demonstrated that receptors can be applied to the isolation of ligands from clean sample extracts in sample handling on basis of biological recognition. However, the low recovery of DES in the presence of 17βES is the result of competition between the two ligands in equilibrium for a limited number of receptor binding sites. An excess of receptor binding sites will eliminate competition. Furthermore, immobilization of a high amount of ER onto a solid support for the use in a dynamic sample handling system will probably contribute to an increase in the rate of association. Affinity constants are high enough to ensure immediate association. However, dissociation of the receptor–ligand complex in a dynamic system should be slow in order to obtain retention of the ligands on the affinity column within the time interval needed for sample handling.

Thus, to obtain an operational screening meth-

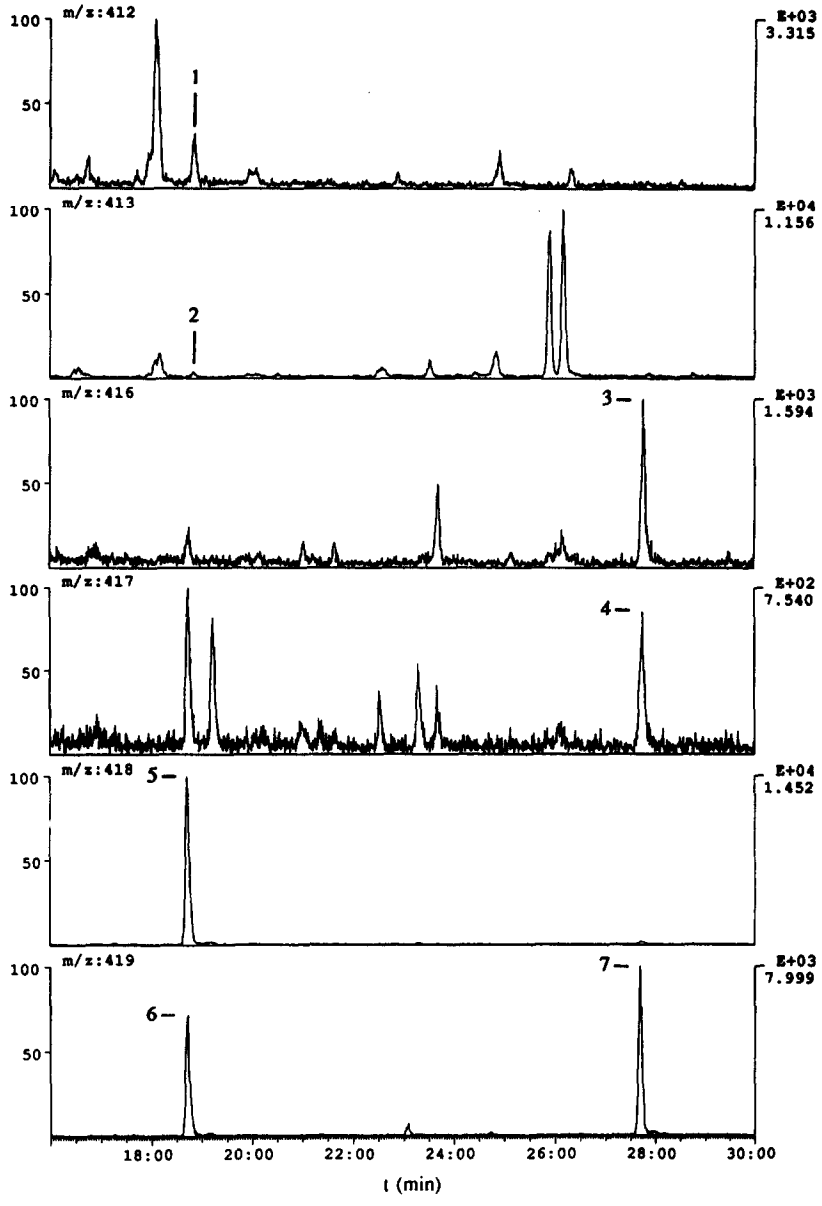

Fig. 2. GC–MS chromatograms by SIM at different m/z ratios of a 1:1 mixture of 17βES and DES (1 ng each) obtained after performing the procedure as described under *Application of the ER in sample handling followed by GC–MS*. Relevant peaks: 1 = DES-TMS; 2 = ^{13}C-DES-TMS; 3 = 17βES-TMS; 4 = ^{13}C-17βES-TMS; 5 = [$^{2}H_6$]DES-TMS; 6 = [$^{2}H_6$]^{13}C-DES-TMS; 7 = [$^{2}H_3$]17βES-TMS.

od for estrogenic compounds a high receptor capacity is required to eliminate competition. This eventually leads to higher recoveries which makes the technique more suitable to be combined with LC. Recombinant DNA techniques should be employed to obtain high amounts of

ER. The development of a screening method for estrogenic compounds in calf urine is under investigation.

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6. References

[1] C.J.M. Arts, P.T.W. Kemperman and H. van den Berg, *Food Addit. Contam.*, 6 (1989) 103–116.

[2] C.D. Banner, A. Goos-Nilsson, J. Sjövall, J.-Å. Gustafsson and J.J. Rafter, *Anal. Biochem.*, 200 (1992) 163–170.

[3] M. Ikeda, S. Watanabe and T. Kusaka, *Steroids*, 52 (1988) 187–203.

[4] G.H. Ingerowsky and H.J. Stan, *Dtsch. Lebensm.-Rundsch.*, 74 (1978) 1–5.

[5] B.H. Durham and I.W. McDicken, *Med. Sci. Res.*, 17 (1989) 525–526.

[6] A.W. Steggles and R.J.B. King, *Biochem. J.*, 118 (1970) 695–701.

[7] R.F. Bruns, K. Lawson-Wendling and T.A. Pugsley, *Anal. Biochem.*, 132 (1983) 74–81.

[8] M. Huang, L. Metherell and O.P. Rorstad, *J. Recept. Res.*, 8 (1988) 831–838.

[9] H.J.G.M. Derks, J. Freudenthal, J.L.M. Litjens, R. Klaassen, L.G. Gramberg and V. Borrias-van Tongeren, *Biomed. Mass Spectrom.*, 10 (1983) 209–214.

[10] G.A. Weiland and P.B. Molinoff, *Life Sci.*, 29 (1981) 313–330.

[11] A.L. Livingston, *J. Toxicol. Environ. Health*, 4 (1978) 301–324.

[12] D.A. Shutt and R.I. Cox, *J. Endocrinol.*, 52 (1972) 299–310.

[13] E.M. Bickoff, *Am. Perfum. Cosmet.*, 83 (1968) 59–64.

[14] C.J.M. Arts, *Ph.D. Thesis*, State University Utrecht, Utrecht, 1992.