Journal of Chromatography, 452 (1988) 419–433
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 489

IMMUNOAFFINITY PRE-COLUMN FOR SELECTIVE ON-LINE SAMPLE PRE-TREATMENT IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY DETERMINATION OF 19-NORTESTOSTERONE

A. FARJAM*, G. J. DE JONG, R. W. FREI and U. A. Th. BRINKMAN
Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)
and
W. HAASNOOT, A. R. M. HAMERS, R. SCHILT and F. A. HUF
State Institute for Quality Control of Agricultural Products, Bornsesteeg 45, 6708 PD Wageningen (The Netherlands)

SUMMARY

A liquid chromatographic column-switching system for automated sample pre-treatment and determination of the anabolic hormone β-19-nortestosterone (β-19-NT) and its metabolite α-19-nortestosterone (19-norepitestosterone) in calf urine is described. The system consists of an immunoaffinity pre-column (immuno pre-column) packed with Sepharose-immobilized polyclonal antibodies against β-19-NT, a second pre-column packed with C_{18} bonded silica and an analytical C_{18} column. Urine (25 ml) is directly loaded on the immuno pre-column, where the analytes of interest are trapped by the immobilized antibodies. Next the analytes are desorbed selectively with a solution containing an excess of the cross-reacting steroid hormone norgestrel and transferred, via the second pre-column, to the analytical column.

The recovery of β-19-NT in spiked urine samples was over 95%. The detection limit was 50 ng/l for a 25-ml urine injection. The system showed no loss of analytical performance over a 6-month period, during which about 100 samples were analysed with the same immuno pre-column. The general applicability of this sample pre-treatment method is discussed.

INTRODUCTION

The determination of trace amounts of analytes in complex samples is a well known problem area. In order to reduce the amount of interfering components and to enrich the analytes of interest sample pre-treatment is necessary in most instances. The use of small pre-columns packed with, *e.g.*, hydrophobic or hydrophilic stationary phases, ion exchangers and metal-loaded phases has gained widespread acceptance in this field[1]. Immunoaffinity chromatography, however, which is based on antibody–antigen interaction and, thus, offers a highly selective retention mechanism in chromatography, has rarely been used for sample pre-treatment. The few examples found in the literature are mentioned below.

0021-9673/88/$03.50

Small columns containing immobilized antibodies have been used for sample clean-up before quantification by bioassay. Giberellins[2], estradiol[3,4] and testosterone[4] were determined in biological matrices such as plant extracts, blood plasma, milk and follicular fluid. In another study, the enantiomers of abscisic acid were purified on immunoaffinity columns before their thin-layer chromatographic separation[5]. Immunoaffinity columns have also been used in off-line sample pre-treatment for column high-performance liquid chromatographic (HPLC) analysis. An immunoaffinity column with immobilized antibodies against indole-3-acetamide has been used for the off-line purification of plant extracts prior to HPLC analysis[6] and a similar approach has been reported for the purification and HPLC determination of cytokinins in plant extracts[7,8]. Monoclonal antibodies have recently been used in a pre-column procedure for the clean-up of tissue extracts in combination with the HPLC determination of chloramphenicol[9]. Only a few on-line immunoaffinity sample pre-treatment systems for HPLC have been published. Phenytoin was determined by injecting a 25-μl plasma sample on a pre-column packed with an antibody immobilized on silica (pore diameter 60 Å). The trapped phenytoin was desorbed by switching the immunoaffinity pre-column on-line with the analytical column[10]. A comparable system was used for the determination of cortisol in serum and urine samples[11].

In this paper we describe an on-line immunoaffinity sample pre-treatment procedure for HPLC, which allows both clean-up and trace enrichment by direct injection of large amounts of urine. The potential of the new technique is demonstrated for the determination of β-19-nortestosterone (β-19-NT) and its metabolite α-19-nortestosterone (or 19-norepitestosterone) (α-19-NT) in calf urine. Their structures are shown in Fig. 1. β-19-NT may be illegally used as an anabolic steroid for the growth promotion of cattle and also as a doping agent in sports. The determination of β-19-NT and α-19-NT in biological samples is extremely difficult, because in cases of abuse the concentrations in samples such as plasma, meat and urine are usually about or below 1 ppb, and also because, *e.g.*, many endogenous steroids interfere with their determination. Consequently, most existing analytical methods are cumbersome[12,13], because they consist of a combination of different analytical procedures, *e.g.*, off-line sample pre-treatment, HPLC and immunoassay. This paper demonstrates that with the help of an immunoaffinity sample pre-treatment step an automated HPLC system can be set up without the need for extensive off-line sample pre-treatment.

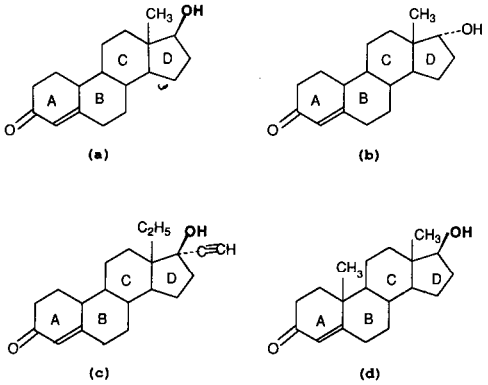

Fig. 1. Structures of (a) β-19-nortestosterone, (b) α-19-nortestosterone, (c) norgestrel and (d) testosterone.

EXPERIMENTAL

Apparatus

The set-up of the HPLC systems used is shown schematically in Fig. 2. HPLC system No. 1 consisted of two Kontron (Zurich, Switzerland) Model 410 pumps, both equipped with laboratory-made membrane pulse dampers, a Kontron MCS 670 Tracer valve switching unit, a Kontron Model 200 programmer, a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 UV detector set at 247 nm, a Kipp & Zonen (Delft, The Netherlands) BD 40 recorder and a Kontron Anacomp Model 220 computer. The analytical column was a laboratory-packed 100 mm × 3 mm I.D. glass column filled with 5-μm LiChrosorb RP-18 (Merck, Darmstadt, F.R.G.), and was protected with a 10 mm × 2 mm I.D. guard column packed with the same material. The 10 m × 2 mm I.D. C_{18} pre-column was packed with Baker (Deventer, The Netherlands) C_{18} 40-μm stationary phase.

HPLC system No. 2 consisted of a Merck–Hitachi (Darmstadt, F.R.G.) Model 655-A-11 pump for the analytical column, a Kratos Model 400 pump for sample handling, two Kratos Model Must valve-switching units, a Kratos Spectroflow 450 solvent programmer, a Merck–Hitachi L-4200 UV–VIS detector set at 247 nm and a Merck–Hitachi Model D 2000 integrator. The analytical column was a Chrompack (Middelburg, The Netherlands) 100 mm × 3 mm I.D. glass column packed with Chromspher 5-μm C_{18} and protected with a Chromsep reversed-phase guard column. The C_{18} pre-column was a 10 mm × 2 mm I.D. Chrompack reversed-phase pre-concentration column.

For details on the immuno pre-column used in both systems, see below.

Chemicals

Sepharose CL-6B and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia (Woerden, The Netherlands). HPLC-grade acetonitrile and

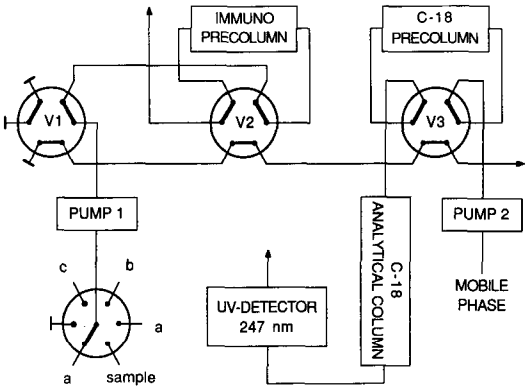

Fig. 2. Set-up of the automated HPLC system for the determination of β-19-NT and α-19-NT in urine samples. Immuno pre-column, 10 mm × 10 mm I.D. stainless-steel column packed with Sepharose-immobilized antibodies; C_{18} pre-column, 10 mm × 2 mm I.D. stainless-steel column. a, Water; b, acetonitrile–water (5:95) containing 190 μg/l of norgestrel; c, methanol–water (70:30). Mobile phase, acetonitrile–water (35:65); flow-rate of pump 1, 10 ml/min for flushing the capillaries (steps 2, 4, 6 and 8 in Table II) and 4 ml/min for all other steps; flow-rate of pump 2, 0.7 ml/min. Valves V1–V3 are all shown in position A (compare Table II).

methanol were obtained from Baker and Merck. HPLC-grade water was prepared from demineralized water using a Milli-Q (Millipore, Bedford, MA, U.S.A.) water purification system with subsequent filtration through an HPLC column filled with 40-μm Baker C_{18} material. The eluents were degassed under vacuum in an ultrasonic bath. β-19-NT, norgestrel (NG; see Fig. 1c) and testosterone (see Fig. 1d) were obtained from Sigma (St. Louis, MO, U.S.A.); α-19-NT was a gift from Organon (Oss, The Netherlands). Stock solutions of the steroids were prepared in ethanol (1 mg/ml) and stored at 4°C until use.

Norgestrel was purified by preparative HPLC; 1 mg of norgestrel was dissolved in 200 μl of methanol and injected on to a Hibar (Merck) 250 mm × 10 mm I.D. LiChrosorb RP-18 column. Acetonitrile–water (55:45) was used as the mobile phase.

All other chemicals were of analytical-reagent grade.

Antibody preparation and immobilization

Steroid hormones are too small to be immunogenic and must therefore be conjugated to a molecule large enough to elicit an immune response. In this study, a 19-nortestosterone-17β-hemisuccinate–BSA (bovine serum albumin) conjugate, synthesized according to Kyrein[14], was used for the immunizaton of a rabbit. The collected antiserum was purified according to the method of Steinbuch and Audrian[15]. The isolated IgG (immunoglobulin G) fraction was stored freeze-dried (RIKILT batch 726-3). Cross-reactivities, as determined by radioimmunoassay, were *ca.* 70%, 20% and less than 1% for α-19-NT, NG and testosterone, respectively.

The IgG fraction was immobilized on cyanogen bromide-activated Sepharose 4B as recommended by the manufacturer[16]. Two different amounts of IgG were used for immobilization. In batch A 0.6 mg of freeze-dried IgG was used per ml of gel and in batch B 10 mg per ml of gel. For the latter batch the coupling efficiency was 46%, as determined by measurement (UV at 280 nm) of non-bound IgG in the coupling solution before and after immobilization. The immunosorbents obtained were stored in 0.1 *M* phosphate buffer (pH 7.2) containing 0.02% sodium azide.

Packing of immunoaffinity pre-columns

Laboratory made 10 mm × 10 mm I.D. or 10 mm × 4 mm I.D. stainless-steel columns, equipped with 5-μm stainless-steel screens and PTFE rings as the column inlet and outlet were used. For packing the screens and PTFE rings were removed at one end and the pre-columns were connected to a vacuum facility with their closed ends. A thick slurry of immunosorbent in storage buffer was filled into the pre-column, then reduced pressure was applied in order to obtain a higher packing density, and the void thus created was packed with additional immunosorbent. The pre-column was closed under reduced pressure. Next the column was connected to an HPLC pump and flushed successively with 50 ml of 0.1 *M* phosphate buffer (pH 7.0), 50 ml of demineralized water and 50 ml of methanol–water (70:30) at a flow-rate of 4 ml/min (2 ml/min for the 10 mm × 4 mm I.D. pre-column). The column was stored in methanol–water (70:30) at 4°C or at room temperature.

Determination of β-19-NT binding capacity

The binding capacity of the immunoaffinity pre-columns (immuno pre-columns) for β-19-NT was determined by pre-concentrating 16 ml of a standard solution

containing 100 μg/l of β-19-NT and performing the analysis according to the final procedure discussed below. From the β-19-NT peak area the binding capacity was calculated by comparison with a standard loop injection. Using a 10 mm × 10 mm I.D. pre-column the capacity was 60 ng for immunosorbent A and 340 ng for immunosorbent B when acetonitrile–water (10:90) was used for desorption. When desorption was performed with acetonitrile–water (5:95) containing 190 μg/l of norgestrel, immunosorbent B showed a capacity of 450 ng (for an explanation, see below).

RESULTS AND DISCUSSION

Stationary phase for immobilization of antibodies

An important aspect of the design of an on-line immunoaffinity sample pretreatment system is the nature of the support material used for the immobilization of the antibodies. In order to obtain good selectivity, the support should show low non-specific interactions with the constituents of the biological samples. In this respect, polysaccharide stationary phases such as agarose are the best material available. Their only disadvantage is their compressibility, which makes them less suitable for high-pressure systems.

Experiments with the agarose gel Sepharose CL-6B, packed in a 10 mm × 4 mm I.D. pre-column, showed that no collapse of the stationary phase occurred at flow-rates below 3.5 ml/min. The packed pre-column turned out to be stable, even when the mobile phase was switched from water to a phosphate buffer or methanol–water (70:30), or to filtered urine, and *vice versa*. In other words, agarose-based pre-columns can be loaded, flushed and desorbed without problems.

Desorption from the immuno pre-column: technical aspects

Initial experiments showed that with the agarose-based pre-column desorption could not be realized by simply switching the pre-column on-line with an analytical column (100 mm × 3 mm I.D., 5-μm stationary phase). The agarose pre-column became blocked immediately, even if the flow-rate through the analytical column was as low as 0.7 ml/min. Probably the sudden pressure change generated by the switching caused the agarose gel to collapse. In order to circumvent this problem, a dual pre-column system was designed (Fig. 2), in which the agarose pre-column is switched in series with a second pre-column during the desorption step. The small dimensions of the second pre-column (10 mm × 2 mm I.D.), combined with the large particle diameter (40 μm) of its C_{18} bonded silica stationary phase, result in a very low back-pressure, thereby avoiding a large pressure shock during the desorption of the agarose-based pre-column. In a second switching step, the analytes of interest are transferred from the second pre-column to the analytical column.

Desorption from the immuno pre-column: chemical aspects

Analyte desorption with aqueous solutions. For optimum performance, desorption from the large-volume immuno pre-column must be carried out with a solvent, which allows peak compression on the second, reversed-phase, pre-column. Therefore, several purely aqueous solutions were examined, which are frequently used for desorption purposes in affinity chromatography[16].

None of aqueous 2.5 *M* sodium thiocyanate, 1 *M* propionic acid (pH 3.0), 0.1 *M* citrate buffer (pH 3.0), 0.7 *M* trichloroacetic acid (pH 2.3) or ethylene glycol–water (20:80) was suitable for desorption, because impurities were present in these solutions which completely obscured the β-19-NT peak in the chromatograms. Other solutions passed this test, such as 0.1 *M* phosphate buffer (pH 6.0 or 8.0), 0.1 *M* acetate buffer (pH 3.4, 4.6 or 5.7), 1 *M* sodium chloride and 0.1 *M* glycine–hydrochloric acid buffer (pH 3.0); however, they were not able to desorb β-19-NT at all; in each instance, the recovery of β-19-NT was zero. Experiments which were performed at a later stage using the final procedure showed that β-19-NT can even be pre-concentrated with good recovery from these solutions on the immuno pre-column!

The above results agree with those reported by Davis *et al.*[7], who unsuccessfully tried to desorb low-molecular-weight plant hormones from an immunoaffinity column, using either chaotropic ions (ions which disrupt the structure of water and reduce hydrophobic interaction), high salt concentrations or changes in pH.

The various aqueous solutions, which have been successfully applied for many years for the desorption of proteins from immunoaffinity columns, obviously fail to desorb small molecules. Probably, with these solutions, the desorption of proteins is based mainly on changes in the structure of the bound protein (partial denaturation), and not on radical changes in the structure of the immobilized antibodies. This model is further supported by the fact that irreversible denaturation of the protein during the elution step is a frequently encountered problem in affinity elution. However, no case is known, so far, where the immobilized antibodies have been denatured irreversibly by these aqueous solutions. Because of their multi-point attachment to the stationary phase, the immobilized antibodies are much more resistant to denaturation and, hence, to changes in their structure, compared with free antibodies. Therefore, the elution of small molecules, that is, molecules which are not sensitive to denaturation, needs much more rigorous conditions.

Analyte desorption with aqueous solutions containing organic modifiers. In the literature, pure methanol[6,7], methanol–water (90:5)[4] and acetone–water (95:5)[3] have been used successfully for the desorption of low-molecular-weight compounds from an immunosorbent. In all instances the immunosorbent could be used repeatedly without a significant loss of activity.

In off-line experiments, methanol–water (70:30) was used to desorb trapped β-19-NT from the immuno pre-column; 5 ml of solution sufficed to recover β-19-NT, pre-concentrated from 16 ml of a 1 μg/l standard solution, with a 98–100% recovery (10 mm × 10 mm I.D. immuno pre-column). Unfortunately, this simple procedure could not be used for on-line desorption. The high methanol content did not allow the reconcentration of the desorbed analyte on the C_{18} bonded silica pre-column. Therefore, lower organic modifier concentrations which would still allow a refocusing of analytes on the reversed-phase system were tested, first with immunosorbent A. When using dioxane–water mixtures several impurities showed up, some of which interfered with the β-19-NT peak. Good results were obtained with methanol–water and acetonitrile–water mixtures. In this study acetonitrile was preferred to methanol because it is also used as a mobile phase constituent. For on-line desorption the highest recovery was achieved by desorbing the immuno pre-column with 50 ml of acetonitrile–water (10:90). For the pre-concentration of 16 ml of a standard solution

TABLE I

RECOVERY OF β-19-NT AS A FUNCTION OF THE COMPOSITION OF THE DESORBING SOLUTION

For further explanation and details, see the text and Table II; desorption here was performed with 50 ml of solution in the back-flush mode.

Composition of the desorbing solution	*β-19-NT recovery (%)*
Immunosorbent A (0.6 mg/ml):	
Acetonitrile–water (5:95)	< 1
Acetonitrile–water (10:90)	85–90
Acetonitrile–water (15:85)	60
Immunosorbent B (10 mg/ml):	
Acetonitrile–water (10:90)	< 5
190 μg/l NG in water	70
190 μg/l NG in acetonitrile–water (5:95)	95–100
38 μg/l NG in acetonitrile–water (5:95)	78

(1 μg/l β-19-NT) the recoveries were 85–90% ($n=4$) (Table I; recoveries calculated by comparison with standard loop injection). The use of a lower percentage, *i.e.*, 5%, of acetonitrile gave a recovery of less than 1% β-19-NT after desorption with 50 ml of the eluent. Elution with acetonitrile–water (15:85) led to a recovery of only 60%, which was probably due to breakthrough on the C_{18} pre-column.

Using immunosorbent A, with its relatively low IgG concentration, the quoted procedure worked well with standard solutions of β-19-NT. However, in order to extend the linear detection range and to reduce the effect of cross-reacting steroids present in real samples, immunosorbent B with its higher amount of immobilized antibodies was used (binding capacity, 340 *vs.* 60 ng for a 10 mm × 10 mm I.D. pre-column). Initial experiments with immunosorbent B, however, gave disappointing results. The recovery, which was 85–90% with immunosorbent A, dropped to less than 5% ($n = 8$) with immunosorbent B (Table I). By subsequent off-line desorption with methanol–water (70:30) it could be shown that the 95% loss of β-19-NT found with immunosorbent B was due to incomplete desorption.

The unexpected behaviour of this immunosorbent can be explained by the nature of the immobilized antibodies. Polyclonal antibodies are, in fact, mixtures of different antibodies. These antibodies (produced by different clones) may have different affinities for the antigen. If an antigen-containing solution is passed through an immunosorbent column, the antigens will first be bound by the antibodies with the highest antigen affinity. Subsequently, the antibody types with lower affinities become saturated with antigen. The phenomenon described above may be related to antigens bound to high-affinity antibodies, which are not eluted with acetonitrile–water (10:90). Because of the higher IgG content of immunosorbent B, more β-19-NT is bound to the high-affinity antibodies. This model can explain the divergent recoveries.

Immunoselective analyte desorption using a competing steroid. For quantitative desorption of β-19-NT from the immuno pre-column, a more specific principle is desired. The use of a compound with a structure similar to that of β-19-NT, which

can act as a displacer by competing for the high-affinity sites present in the immunosorbent, was tested. The synthetic steroid hormone norgestrel (NG; Fig. 1c) seemed to have a suitable structure for this purpose. On the one hand, NG has the same structure in the A and B rings as has β-19-NT, and hence is a good candidate to show a high affinity to the immunosorbent (the A and B rings are important for recognition because β-19-NT was conjugated to BSA at the opposite part of the molecule for immunization). On the other hand, in contrast to β-19-NT, NG has different substituents in the D ring (the part of the molecule which is not significant for the affinity recognition), which allows an easy HPLC separation from β-19-NT. A displacer solution of 190 μg/l NG in water caused desorption of 70% of the trapped β-19-NT (Table I). The data in Table I clearly show the benefit of the immunoselective desorption which can be achieved with purely aqueous solutions of NG.

In principle two methods can be used to increase further the recovery of 70%: the use of larger desorption volumes or of higher amounts of NG. The disadvantages are a longer analysis time and disturbances caused by the large NG peak, respectively. A more elegant way to increase the recovery was the addition of 5% of acetonitrile to the aqueous NG-containing solution. This resulted in recoveries of 95–100%, probably owing to the reduction of non-specific interactions. Using a lower NG concentration led to inferior results (Table I).

The previous results were achieved by desorbing the immuno pre-column in the forward-flush mode. In order to reduce the desorption volume and hence to shorten the analysis time, back-flush desorption was also tested. The experimental data are given in Fig. 3. A virtually quantitative recovery was achieved with a desorption volume of 33 ml instead of 50 ml. The lower recovery for desorption volumes of over 50 ml is probably due to breakthrough on the C_{18} pre-column.

The immunoselective desorption has an additional advantage over the non-selective desorption with a high percentage of organic modifier. The competing steroid (here NG) will desorb analytes only from the antibodies for which it possesses a high affinity itself. In principle, no desorption will occur from other types of antibodies present in the immunosorbent. This is an important aspect of the present clean-up procedure, because in our case a crude IgG mixture, not purified by affin-

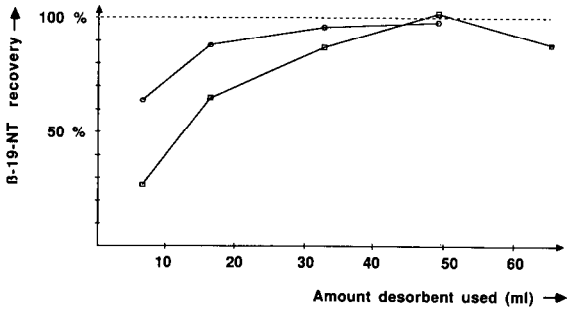

Fig. 3. Recovery of β-19-NT as a function of the amount of acetonitrile–water (5:95) containing 190 μg/l of NG used for the transfer of analytes from the immuno pre-column to the C_{18} pre-column. A 16-ml volume of a 1 μg/l β-19-NT standard solution was pre-concentrated and analysed according to the schedule in Table II. Forward-flush desorption (□) is compared with back-flush desorption (○). For back-flush desorption the system was set up according to Fig. 2. HPLC system No. 1 was used for the measurements.

ity-based techniques, was used for immobilization, and large amounts of antibodies of unknown specificity are present in such an immunosorbent. Further, interferences by compounds which are bound non-specifically to the stationary phase backbone, the coupling group, other immobilized proteins or even the large non-selective surface of the antibodies, will be much reduced if the selective desorption technique is utilized.

Final procedure

The final analytical procedure is summarized in Table II (also see Fig. 2). The first step involves the preconditioning of the immuno pre-column with water. Then the sample is introduced by pump 1 via the solvent selection valve. Next, the immuno pre-column is flushed with water to displace the remaining sample (urine) and to remove non-specifically bound impurities. In the following step, the C_{18} pre-column is switched off-line with respect to the analytical column and preconditioned with water. Subsequently, the immuno pre-column, which now contains the trapped analytes, and the C_{18} pre-column are switched in series and the transfer of the analytes is accomplished in the back-flush mode with an aqueous solution containing 190 μg of NG/l and 5% acetonitrile.

After the complete transfer of the analytes to the C_{18} pre-column, the actual separation is started by switching the C_{18} pre-column on-line with the analytical column; β-19-NT and NG are then separated on the analytical column. Simultaneously, the immuno pre-column is reconditioned by flushing with methanol–water (70:30). In this step NG, which saturates the immuno pre-column during the desorption step, and other compounds which are retained by non-specific interaction, are

TABLE II

SCHEDULE OF THE AUTOMATED ANALYSIS

For each valve, position A corresponds to the position shown in Fig. 2. For further explanation, see text.

Step	*Event*	*Valve position*		
		Valve 1	*Valve 2*	*Valve 3*
1	Flushing immuno pre-column (10 mm × 10 mm I.D.) with 15 ml of water	A	A	A
2	Flushing capillaries with sample	B	A	A
3	Flushing immuno pre-column with sample	A	A	A
4	Flushing capillaries with water	B	A	A
5	Flushing immuno pre-column with 15 ml of water	A	A	A
6	Flushing capillaries with water	B	A	A
7	Flushing C_{18} pre-column with 5 ml of water	B	A	B
8	Flushing capillaries with desorbing solution containing 190 μg/l of NG and 5% of acetonitrile	B	B	B
9	Flushing immuno pre-column and C_{18} pre-column in series with 33 ml of a solution containing 190 μg/l of NG and 5% of acetonitrile	A	B	B
10	Desorbing C_{18} pre-column by on-line switching with the analytical column	A	B	A
11	Flushing immuno pre-column with 20 ml of methanol–water (70:30)	A	A	A

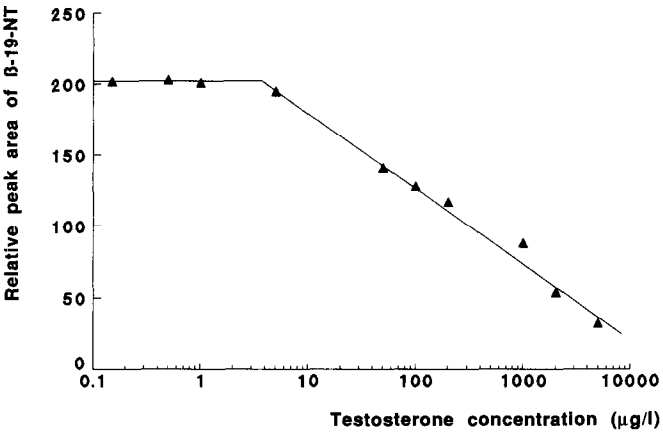

Fig. 4. Recovery of β-19-NT as a function of the testosterone concentration in the sample. A 25-ml volume of a standard solution containing 1 μg/l of β-19-NT and the indicated concentration of testosterone was analysed according to the schedule in Table II, with a 10 mm × 4 mm ID. pre-column instead of the 10 mm × 10 mm I.D. pre-column containing immunosorbent B. HPLC system No. 2 was used for the measurements.

quantitatively desorbed. Now the next analysis can be started, while the separation in the analytical column is still running.

Cross-reactivity of the immunosorbent with α-19-NT and testosterone

In cases of β-19-NT abuse, a metabolite, α-19-NT, is also present in the urine. Both compounds have the same structure in the significant A and B rings of the steroid skeleton (Fig. 1), so, as expected, pre-concentration of α-19-NT standard solutions showed the same high recovery as was achieved with β-19-NT. In other words, the immunosorbent has a similar affinity for both steroids. Fortunately, both compounds can be separated on the analytical column and hence the simultaneous determination of α-19-NT and β-19-NT creates no problems (see Fig. 8).

In addition, the structurally related steroid hormone testosterone (see Fig. 1d) occurs endogenously in urine. Concentrations of up to 2 μg/l have been measured by us in calf urine using gas chromatography–mass spectrometry. Testosterone can interfere in the determination of β-19-NT, although the cross-reactivity of the immunosorbent for testosterone is below 1% (see Experimental). Fig. 4 shows the effect of adding increasing amounts of testosterone to standard solutions of β-19-NT (1 μg/l) on the recovery of β-19-NT. Up to a concentration of about 5 μg/l of testosterone no effect can be seen on the recovery of β-19-NT. Higher testosterone concentrations reduce the recovery of β-19-NT, but the effect is fairly weak (note the logarithmic scale). Even with the small 10 mm × 4 mm I.D. pre-column, packed with immunosorbent B, the recovery of β-19-NT dropped by only 10% at the high testosterone level of 10 μg/l, and by 25% at the unrealistically high concentration of 50 μg/l. This clearly demonstrates that the affinity of the immunosorbent for testosterone is much lower than for β-19-NT.

TABLE III

ANALYTICAL DATA ON THE AUTOMATED ANALYSIS OF β-19-NT-SPIKED CALF URINE SAMPLES

The samples were analysed under the same conditions as in Fig. 5.

Criterion	*Level*	*Result*
Repeatability	1 μg/l β-19-NT ($n=3$)	1.8%
Recovery	1 μg/l β-19-NT ($n=3$)	97%
Linearity	0.2–10 μg/l ($n=5$)	0.9997
Detection limit	S/N* = 3:1 (from Fig. 6)	50 ng/l (1.25 ng)
Total analysis time including sample pre-treatment		45 min

* Signal-to-noise ratio.

Performance and application in spiked urine analysis

The data in Table III clearly demonstrate the potential of the method. Fig. 5 shows a calibration plot, obtained with immunosorbent B, for β-19-NT-spiked urine samples. It is linear for β-19-NT concentrations of 0.2–10 μg/l (r = 0.9997). At higher concentrations the recovery rapidly diminishes, as is to be expected because the total capacity of the immuno pre-column was about 370 ng in this instance.

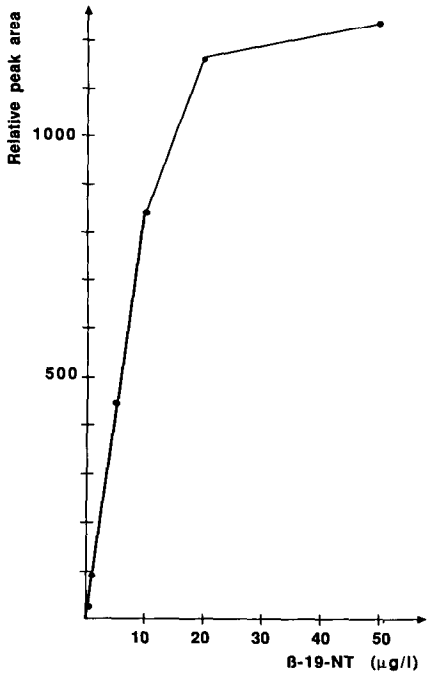

Fig. 5. Peak area as a function of the β-19-NT concentration in spiked urine. Calf urine samples were spiked with β-19-NT, diluted with an equal amount of water and filtered. An aliquot of 50 ml (containing 25 ml of urine) was loaded on immunosorbent B and processed according to the final procedure. HPLC system No. 1 was used for the measurements. The total β-19-NT capacity of the 10 mm × 10 mm I.D. immuno pre-column was 370 ng in this instance.

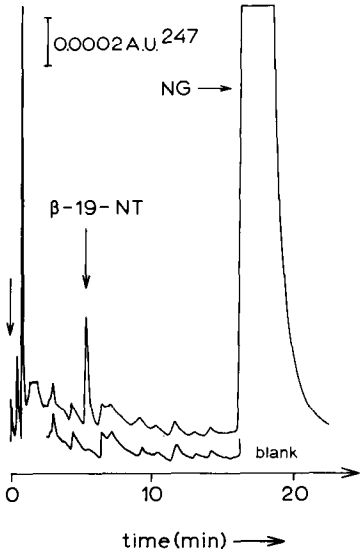

Fig. 6. Chromatogram of a calf urine sample spiked with 200 ng/l of β-19-NT and of the corresponding blank urine. The samples were filtered and diluted with an equal amount of water. An aliquot of 50 ml (containing 25 ml of urine) was loaded on immunosorbent B and processed according to the final procedure. HPLC system No. 2 was used for the measurements.

Fig. 6 shows the chromatogram of a blank calf urine and of the same sample spiked with 200 ng/l of β-19-NT; only peaks due to β-19-NT and NG are observed. In other urine samples, however, several impurities showed up, which sometimes interfered with the detection of small amounts of β-19-NT. In such an event, the interferences can be reduced by diluting the sample with water before loading it on to the system. This is demonstrated in Fig. 7a and b, where a spiked (100 ng/l) urine sample was loaded on to the system either (a) undiluted or (b) after dilution with 4 volumes of water. An inferior improvement was observed on addition of 5% acetonitrile to the urine sample before analysis (data not shown). The results of the combined approach are shown in Fig. 7c. Here the urine was spiked with 2 μg/l of β-19-NT and 20 ml of urine diluted with 20 ml of acetonitrile–water (10:90) were loaded. The interferences were reduced to a similar extent as was reached with 4-fold dilution with water and, additionally, the analysis time was shortened considerably. Besides, even though acetonitrile was added to the sample, no decrease in recovery was observed. The chromatogram of another "dirty" urine sample, which was processed similarly and had been spiked withh 0.3 μg/l each of β-19-NT and α-19-NT, is shown in Fig. 8. The resolution of both peaks is very satisfactory.

If a sample volume of 25 ml is loaded, the detection limit for β-19-NT is 50 ng/l in spiked urine (Table III). In principle, this value can be improved by increasing the sample volume. Further, the sample can be loaded at a higher flow-rate to reduce the time of analysis. In fact, the same high β-19-NT recovery was recorded when the sample from Fig. 7b was loaded at a flow-rate of 10 ml/min instead of 4 ml/min. In other words, the antibody–antigen interaction is fast enough to permit such high flow-rates.

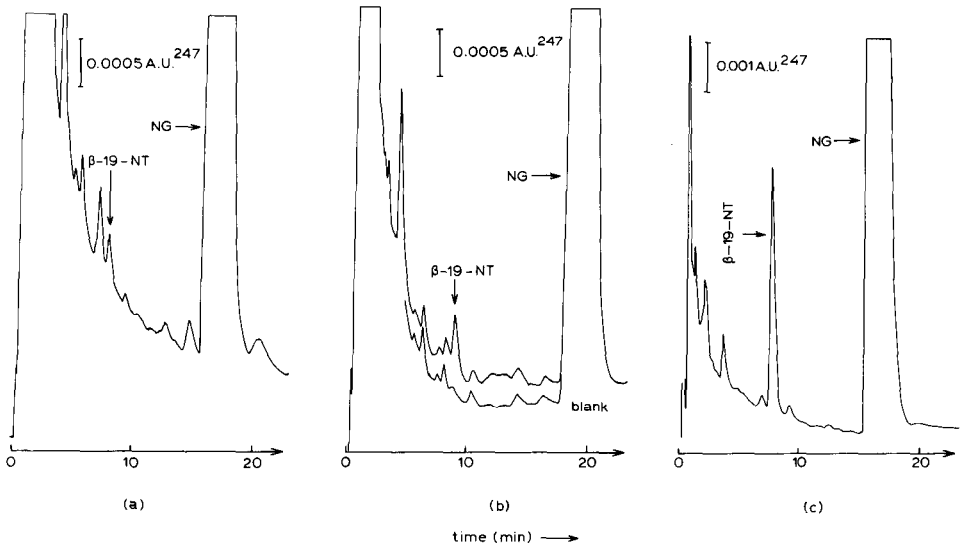

Fig. 7. Chromatograms of a spiked, and filtered, calf urine sample. (a) 36 ml of urine spiked with 100 ng/l of β-19-NT, pre-concentrated without dilution; (b) 36 ml of urine spiked with 100 ng/l of β-19-NT, diluted to 180 ml with water and pre-concentrated; (c) 20 ml of urine spiked with 2 μg/l of β-19-NT, diluted with 20 ml of acetonitrile–water (10:90) and pre-concentrated. The analysis was performed according to the final procedure. HPLC system No. 1 was used for the measurements.

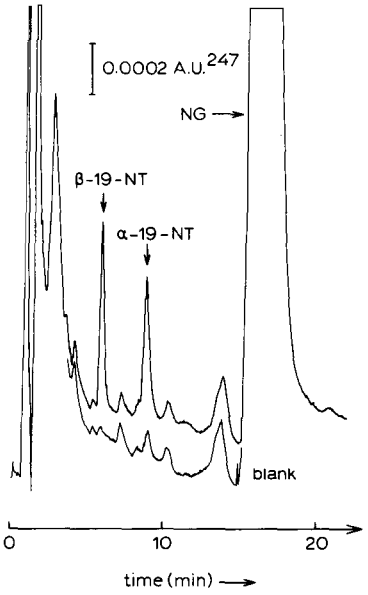

Fig. 8. Chromatograms of a calf urine sample spiked with 300 ng/l each of β-19-NT and α-19-NT and of the corresponding blank urine. The samples were filtered and diluted with an equal amount of acetonitrile–water (10:90). An aliquot of 53 ml (containing 26.5 ml of urine) was loaded and processed according to the final procedure. HPLC system No. 2 was used for the measurements.

Capacity, stability and storage of the immuno pre-columns

The capacity of the immuno pre-columns decreased slowly during their use. Over a 6-month period about 100 urine samples were analysed, using various analytical conditions, *i.e.*, testing different solvent mixtures. During this period the capacity of a 10 mm × 10 mm I.D. precolumn packed with immunosorbent B decreased from 450 to 180 ng which was, however, sufficient to determine β-19-NT in the desired concentration range. Losses were not observed during storage for, *e.g.*, 30 days, but only when the column was used for analysis. After an initial rapid decrease in capacity, the losses became much smaller once a few analyses had been carried out. Ligand leakage is probably the reason for this behaviour. It is well known that cyanogen bromide-coupled ligands are not very resistant to leakage. The use of other coupling reactions may help to improve the stability of the immunosorbent.

When not in use, the immunosorbents were stored in methanol–water (70:30) at 4°C or at room temperature. As this is the same solvent as is used in the last step of the analysis, no extra flushing step is needed to prepare the column for storage. Moreover, the addition of chemicals to prevent microbial degradation can be omitted.

CONCLUSIONS

An immunoaffinity separation is, by nature, a digital separation step rather than a continuous chromatographic procedure; that is, in principle capacity factors are close either to infinity or to zero. This is exactly what is needed in sample preparation. Because of the large retention that can be achieved in immunoaffinity chromatography, the early breakthrough of analytes that often complicates conventional pre-column procedures hardly plays a role here. In addition, the selectivity of the immunosorbent creates an extremely high clean-up efficiency.

In this paper, the potential of on-line immunoaffinity-based sample preparation for HPLC analysis has been demonstrated. The analytes of interest were concentrated on a pre-column packed with a soft immunosorbent gel displaying low non-specific interaction. The on-line desorption turned out to be a critical step and was finally performed with an almost purely aqueous solution containing a large excess of a competing steroid. The total analytical system displays considerable selectivity, which is mainly due to the pre-column antibody–antigen interaction and to the selective immunochemical desorption. At least 100 large-volume (25 ml) urine samples can be analysed without exchanging the immuno pre-column. The total time of analysis, including the automated sample pre-treatment, is about 45 min. If a large-volume autosampler or, even simpler, a multi-port solvent selection valve is available, the system can be operated unattended. The detection limit of 50 ng/l of β-19-NT for spiked urine samples is in the range required for the free drug in veterinary and doping analysis. The possibility of determining not just the free drug, but also its glucuronide and sulphate conjugates, which are present in urine at much higher concentrations, either by off-line hydrolysis or on-line by immobilized enzyme reactors, is currently being investigated. The method will also be applied to faeces, meat and bile samples.

Compared with immunoassay methods, the present system has the advantages that (i) cross-reacting compounds can be distinguished from the analytes, (ii) the

reproducibility is better, (iii) full automation can be achieved easily and (iv) more than one analyte can be determined in one run. In principle, the method can be applied to all compounds which elicit an immune response. Current research is aimed at demonstrating this. Further, combining several types of antibodies in one pre-column or, more elegantly, combining various immuno pre-columns in series, offers the possibility of determining even more analytes in just one run, and allows in principle the construction of a fully automated analytical system, the selectivity of which can be tailor-made by selecting a suitable set of immuno pre-columns. In the near future, our main attention will be focused on solving some of the analytical and technical challenges outlined here and, in addition, on investigating the mechanism of the immunoselective desorption in order to create a better model for its understanding and optimization.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Foundation for Technical Science, grant No. VCH 46.0616. The National Inspection Service for Meat, Livestock and Animal Products is thanked for technical support. Organon (Oss, The Netherlands) is thanked for the gift of α-19-nortestosterone. The initial assistance of Euro-Diagnostics (Apeldoorn, The Netherlands) is highly appreciated.

REFERENCES

1 R. W. Frei and K. Zech (Editors), *Selective Sample Handling and Detection in High-Performance Liquid Chromatography*, Elsevier, Amsterdam, 1988.
2 Y. Fuchs and E. Gertman, *Plant Cell Physiol.*, 15 (1974) 629–633.
3 R. G. Glencross, S. A. Abeywardene, S. J. Corney and H. S. Morris, *J. Chromatogr.*, 223 (1981) 193–197.
4 R. Webb, G. Baxter, D. McBride, G. D. Nordblom and M. P. K. Shaw, *J. Steroid Biochem.*, 23 (1985) 1043–1051.
5 E. M. S. MacDonald and R. O. Morris, *Methods Enzymol.*, 110 (1985) 347.
6 P. Ulvskov, J. Marcussen, R. Rajagopal, E. Prinsen, P. Ruedelsheim and H. Van Onckelen, *Plant Cell Physiol.*, 28 (1987) 937–945.
7 G. C. Davis, M. B. Hein, D. A. Chapman, B. C. Neely, C. R. Sharp, R. C. Durley, D. K. Biest, B. R. Heyde and M. G. Carnes, *Plant Growth Substances*, Springer, Berlin, 1985, pp. 44–51.
8 G. C. Davis, M. B. Hein and D. A. Chapman, *J. Chromatogr.*, 366 (1986) 171–189.
9 C. van de Water and N. Haagsma, *J. Chromatogr.*, 411 (1987) 415–421.
10 B. Johansson, *J. Chromatogr.*, 381 (1986) 107–113.
11 B. Nilsson, *J. Chromatogr.*, 276 (1983) 413–417.
12 E. H. J. M. Jansen, R. H. van den Berg, G. Zomer, R. Both-Miedema, C. Enkelaar-Willemsen and R. W. Stephany, *Anal. Chim. Acta*, 170 (1985) 21–27.
13 C. van Peteghem, *J. Chromatogr.*, 369 (1986) 253–257.
14 H. J. Kyrein, *Z. Lebensm. Unters. Forsch.*, 177 (1983) 426–438.
15 M. Steinbuch and R. Audrian, *Arch. Biochem. Biophys.*, 134 (1969) 279–284.
16 *Affinity Chromatography, Principles and Methods; Product Information*, Pharmacia, Uppsala, 1983.