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Review

High-performance liquid chromatographic separation and detection methods for anabolic compounds

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ABSTRACT

The role of high-performance liquid chromatography (HPLC) in methods of analysis for anabolic compounds in biological samples is reviewed. Special attention is given to both the separation and detection of anabolic compounds. A distinction is made between on-line detection systems, such as ultraviolet detection and diode-array detection, and off-line detection methods with special emphasis on immunochemical detection methods using non-isotopic labels. A number of applications are given to elucidate the possibilities of HPLC in the analysis of anabolic compounds.

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LIST OF ABBREVIATIONS

ABEI	N-(4-Aminobutyl)-N-ethylisoluminol
DE	Dienestrol
DES	Diethylstibestrol
E2	17β-Estradiol
EE2	Ethinylestradiol
GC–MS	Gas chromatography–mass spectrometry
HEX	Hexestrol
HPLC	High-performance liquid chromatography
NT	19-Nor-17β-testosterone
MP	Medroxyprogesterone
MT	17α-Methyltestosterone
P	Progesterone
RIA	Radioimmunoassay
T	17β-Testosterone
TB	Trenbolone
TD	Trendione
TLC	Thin-layer chromatography
Z	Zeranol

1. INTRODUCTION

In the EEC, the use of xenobiotic anabolic agents in food-producing animals is prohibited by law. To ban the use of these compounds effectively, a good and efficient control programme is needed in which validated analytical methods are used [1]. In addition to immunochemical screening methods and expensive confirmatory methods such as gas chromatography–mass spectrometry, high-performance liquid chromatography (HPLC) has proved to be a useful separation and detection method in the forensic analysis of anabolic compounds. Especially during the last ten years, HPLC has become increasingly important as a result of the development of new specific and more sensitive detection methods.

The aim of this paper is to review the use of HPLC techniques in the detection and determination of anabolic compounds. Emphasis will be put on the separation of anabolic compounds and specific detection with UV spectroscopic and immunochemical methods. Both methods of analysis and applications will be outlined.

2. HPLC SEPARATION METHODS

HPLC is a very powerful separation technique giving high-resolution separations of closely related compounds, such as anabolic steroids. In addition, HPLC with normal column dimensions can act as a semi-preparative prepurification technique for subsequent spectroscopic analysis. For some purposes HPLC with on-line spectrometric detection can also be used as a powerful and reliable method of analysis. The development of very sensitive UV–VIS detectors and multi-wavelength detection by the diode-array technique has added an additional tool to the analysis of anabolic compounds.

2.1. Reversed-phase separations

Reversed-phase columns are the most com-

monly used, reliable and robust HPLC columns. Reversed-phase materials (*e.g.*, C_{18} and C_8) are very stable and can be used repeatingly without special precautions. Mixtures of water with methanol, acetonitrile or tetrahydrofuran are often used as mobile phases. In most instances an isocratic elution mode is sufficient for the separation of most anabolic compounds. Examples of baseline separations of reversed-phase HPLC have been reported by several workers. Jansen *et al.* [2] reported the separation of several groups of anabolic compounds, such as stilbenes, resorcylic acid lactones (zeranol-like compounds) and a mixture of the most frequently found anabolics [trenbolone (TB), 19-nor-17β-testosterone (NT), zeranol (Z), diethylstilbestrol (DES), 17α-methyltestosterone (MT) and medroxyprogesterone (MP)]. The retention times of a number of anabolic compounds are listed in Table 1. Other more recent lists of HPLC retention times of reversed-phase separations of anabolics have been reported [3,4].

A chemometric approach to the reversed-phase separation of anabolic compounds has been presented by Jansen *et al.* [5]. They used a modified program from Gertz and Fellmann [6] for the calculation of the optimum separation of mixtures of anabolic compounds. In Fig. 1 an example is given of calculated separations of a mixture of eight anabolic compounds. The program is available on request from the authors.

2.2. Normal-phase separations

In addition to reversed-phase separations of anabolic compounds, normal-phase separations have also been published. The use of a Hypersil silica column was reported [7] for the separation of nine of the most often used anabolics. As expected, the elution behaviour was complementary to that of the reversed-phase separation (Table 1). It appeared that not all components were separated with baseline resolution. The reproducibility of the retention times caused some problems because of the influence of very small amounts of water in the mobile phase. The use of

TABLE 1

RETENTION TIMES OF ANABOLIC COMPOUNDS ON THREE DIFFERENT HPLC SYSTEMS AND THE MAXIMUM ABSORPTION WAVELENGTHS

Anabolic compound[a]	Retention time (min)			Maximum wavelength[e] (nm)
	Hypersil ODS[b]	Hypersil silica[c]	LiChrosorb diol[d]	
DES	4.9	7.1	14.5	195 (240)
DE	5.2	7.8	15.7	195 (227)
HEX	5.7	8.5	13.6	195 (225)
Z	4.5	5.9	14.1	215 (262,301)
TB	3.7	4.4	7.1	347 (238)
NT	4.3	5.3	5.4	243
MT	6.5	8.8	3.9	245
MP	8.3	12.7	4.8	245
T	5.3	6.8	4.9	245
E2	4.8	7.3	11.5	197 (220,280)
P	10.2	3.8	2.3	243

[a] Compounds as defined in the text and dienestrol (DE), hexestrol (HEX), 17β-testosterone (T), 17β-estradiol (E2) and progesterone (P).

[b] Mobile phase: methanol–water (65:35, v/v).

[c] Mobile phase: isooctane–methanol (98:2, v/v).

[d] Mobile phase: isooctane–methanol (97:3, v/v).

[e] Submaxima are given in parentheses.

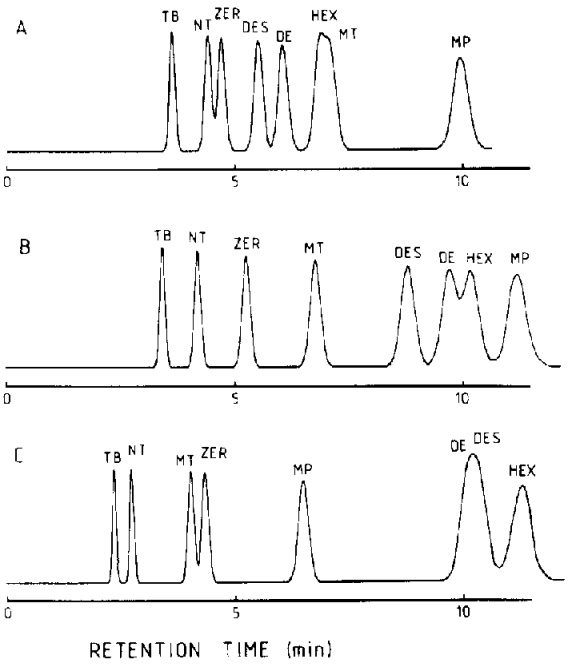

Fig. 1. Schematic HPLC traces of the separation of several anabolics. (A) HPLC separation with the starting solvent system [methanol–water (60:40)]; (B) calculated separation with the solvent system AB–AC (20:80); (C) calculated separation with the solvent system AB–AC–BC (10:10:80). Solvent system AB consisted of a 1:1 mixture of mobile phase A [methanol water (60:40)] and mobile phase B [tetrahydrofuran–water (39.3:60.7)]. Solvent system AC consisted of a 1:1 mixture of mobile phase A and C [acetonitrile–water (45.2:54.8)]. Reprinted from ref. 5.

a more convenient normal-phase column, with diol-modified silica, was reported [8] for a mixture of fifteen anabolic compounds and their metabolites. Although not all components were completely separated, the column could be used to distinguish between estrogenic and androgenic compounds (Table 1). This application has favourable implications for the use of final detection methods, such as thin-layer chromatography (TLC) and gas chromatography–mass spectrometry (GC–MS).

3. HPLC DETECTION METHODS

3.1. Single-wavelength UV detection

Although almost all anabolic compounds (excluding TB and derivatives) show a strong absorption at 200 nm, this wavelength is less suitable for detection because of the increased sensitivity of other unwanted matrix components at this wavelength. Most of the anabolic compounds can be monitored with great sensitivity at a UV wavelength of 240 nm. If a sensitive UV detector is used, the androgens and gestagens can be detected at the 1-ng level. Also, DES has a submaximum at 240 nm, whereas the other stilbenes have different absorbance maxima: (*E,E*)-dienestrol (DE) 230 nm, (*Z,Z*)-DE 260 nm and hexestrol (HEX) 225 nm. Completely different absorption spectra are observed for zeranol- and trenbolone-like compounds. Zeranol shows three absorption maxima (235, 275 and 315 nm, with decreasing intensity), whereas trenbolone and derivatives show their maximum absorption at 350 nm and almost no absorption in the 200–300 nm region. The estrogenic compounds such as 17β-estradiol (E2) and ethinylestradiol (EE2) show their absorption maxima at 280 nm, but with a very low intensity. The applications of HPLC with on-line UV or visible detection depend strongly on the matrix of analysis. For the detection of anabolic compounds in application sites this method is very suitable with respect both to interferences of the matrix and to the sensitivity. In the application sites usually high concentrations are found in a relatively clean matrix. Identification can take place based on a specific retention time of the components or rather by multi-wavelength or diode-array detection. An additional advantage is the fact that often in addition to the hydrolysed parent anabolic compounds also the esters of the anabolic compound can be found and used as an additional tool in the identification procedure.

In urine samples, HPLC with on-line UV VIS detection is limited because of the complex urine matrix. Although prepurification by solid-phase or solvent extraction methods can result in much cleaner samples, UV detection at very low levels can still be difficult. Mostly reversed-phase columns are used for this purpose because they have a high loading capacity. If a urine sample is eluted from a reversed-phase column, during the first few minutes a large number of matrix components elute from the column, as monitored at wavelengths between 200 and 280 nm. After about 6 min, the chromatogram is fairly clean and suitable for UV and/or visible detection of

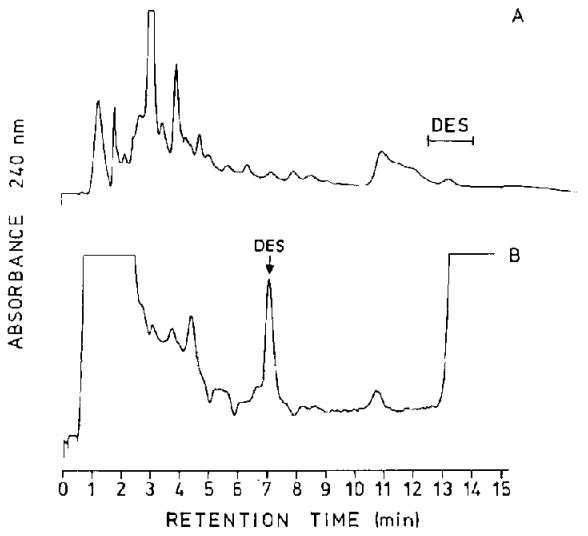

Fig. 2. (A) HPLC of an extract of bovine urine containing 6 μg/l DES on a LiChrosorb diol column. (B) The DES fraction was collected and rechromatographed on a Hypersil ODS column. Reprinted from ref. 9.

the anabolic compounds. An HPLC–UV method to detect *trans*-DES was presented by Jansen *et al.* [9]. They described a two-fold purification procedure involving hexane extraction followed by purification using an HPLC diol system after collection of the *trans*-DES fraction. The final analysis occurred on a reversed-phase HPLC column with UV detection at 240 nm (Fig. 2). A limit of detection of 0.4 ng of DES per injection with a signal-to-noise ratio of 2 was reported, corresponding to urine concentrations down to the 1 ppb (1 μg/l) level.

A simpler prepurification procedure can be used for the detection of TB and its metabolites in urine of veal calves. Because of the optimum detection wavelength of 350 nm, the TB derivatives can be monitored without the interference of urinary matrix components [10]. A simple extraction procedure with diethyl ether is sufficient to obtain clean chromatograms in which TB, its epimer α-trenbolone and its metabolite triendione (TD) can be monitored (Fig. 3). A normal-phase HPLC system was used with isocratic elution with isooctane–ethanol (97:3, v/v). The detection limit was 1–2 μg/l for bovine urine.

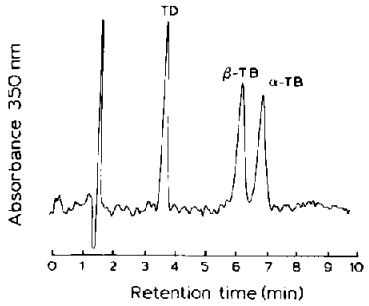

Fig. 3. HPLC of an extract of bovine urine after enrichment with 10 ng of standards of 17α- and 17β-trenbolone and trendione (TD) as indicated. Reprinted from ref. 10.

3.2. Diode-array detection

3.2.1. Methodology

The diode-array detector is a development of the last eight years. The principle is the simultaneous registration of a large number of wavelength intervals, by division of the wavelength scale among a large number of diodes. The information from each diode is stored, which results in complete UV–VIS spectrum collected in a short period of time (50 ms).

The diode-array detection system has in general the folowing possibilities and features. During HPLC analysis a number of UV–VIS spectra (from 200 to 800 nm) can be taken and stored at different times. The times at which spectra will be taken can be programmed before the analysis or can be performed manually during the analysis at a given retention time. The preprogramming can be done in such a way that the system takes spectra automatically at the top, up-slope and down-slope of each peak in the chromatogram. Another possibility is to record the spectra continuously in order to make a three-dimensional plot afterwards.

After the HPLC run, the following possibilities exist for identification and quantitative analysis. Each possibility mentioned hereafter will be illustrated with an example of a mixture of standards of anabolic compounds.

(a) A plot of the chromatogram at a given single wavelength, mainly used for quantification of the components. This possibility is not shown.

(b) A multi-wavelength plot in which a number of chromatograms can be plotted at different wavelengths (Fig. 4).

(c) A three-dimensional plot of the absorbances as function of the retention time and the wavelength (Fig. 5).

(d) A plot of a number of normalized spectra in one plot or the first or second derivatives of the spectra.

(e) A contour plot at a certain level of absorbance as function of retention time and wavelength.

The sensitivity and limits of detection of the anabolic compounds are lower than with a conventional single- or multi-wavelength detector. The limit of detection in the single-wavelength mode (in principle use as a normal UV detector) is in fact not too much lower than with conventional UV detectors. When the diode-array mode is used for spectrum identification, the amount of information makes the detection system less sensitive. Therefore, the low limits of detection as were described for UV detection in the previous section cannot be reached. As a result, the diode-array detection system cannot be used for the on-line determination of anabolic compounds in urine. Most applications have been reported in the identification of anabolic compounds in the so-called injection sites, in which relatively high concentrations of the parent compounds are present.

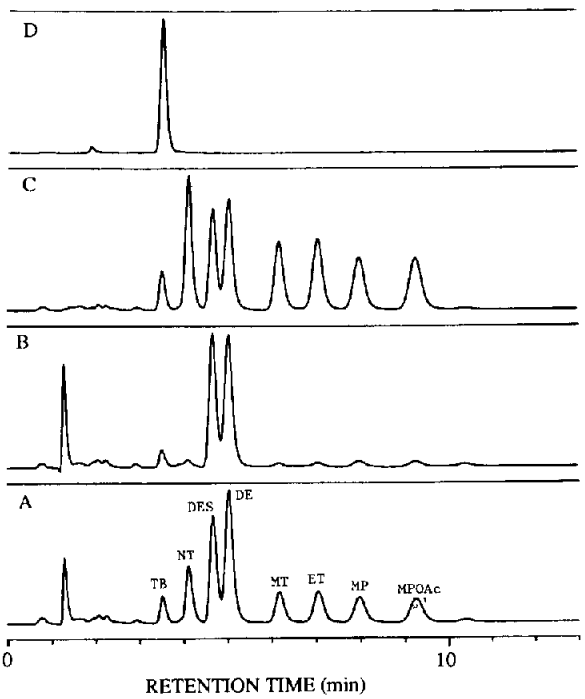

Fig. 4. Multi-wavelength plot for a mixture of anabolic standards [TB, NT, DES, DE, MT, epitestosterone (ET), MP, MP acetate (MPOAc), with increasing retention times, respectively] at (A) 200, (B) 195, (C) 240 and (D) 350 nm.

The specificity of diode-array detection is much better than that of single-wavelength detection. In fact, two specific parameters determine the final specificity: the retention time of the compounds (Table 1) and the total UV–VIS spectrum. In a forensic analysis of anabolic compounds, routinely the spectrum of a standard compound with the same retention time as the unknown component is compared with the spectrum of this component. This is done by plotting both normalized spectra in the same plot. If the standard and the unknown component are identical, a complete match is observed in practice. A combination of these two independent parameters (retention time and complete UV–VIS spectrum) gives a very high level of specific identification in forensic investigations, which is comparable to routine GC–MS analysis.

3.2.2. Applications

As the limits of detection of diode-array detection with full spectra are higher than with single-wavelength detection, urinary concentrations of anabolic compounds are usually too low. Therefore, almost all applications have been described for the analysis and unambiguous identification of anabolic compounds in application sites. Jansen *et al.* [11] reported the UV spectra of the most often used anabolic compounds in a study of 117 samples from application sites. It was shown that a combination of both specific HPLC retention time and a complete UV spectral comparison with standard anabolics results in a fast and very reliable method of detection and identification of anabolics in injection sites of slaughtered animals. An example of an injection site containing DES and NT is shown in Fig. 6.

The assignments performed with the HPLC–diode-array method were compared with the results of GC–MS analyses. Because no discrepan-

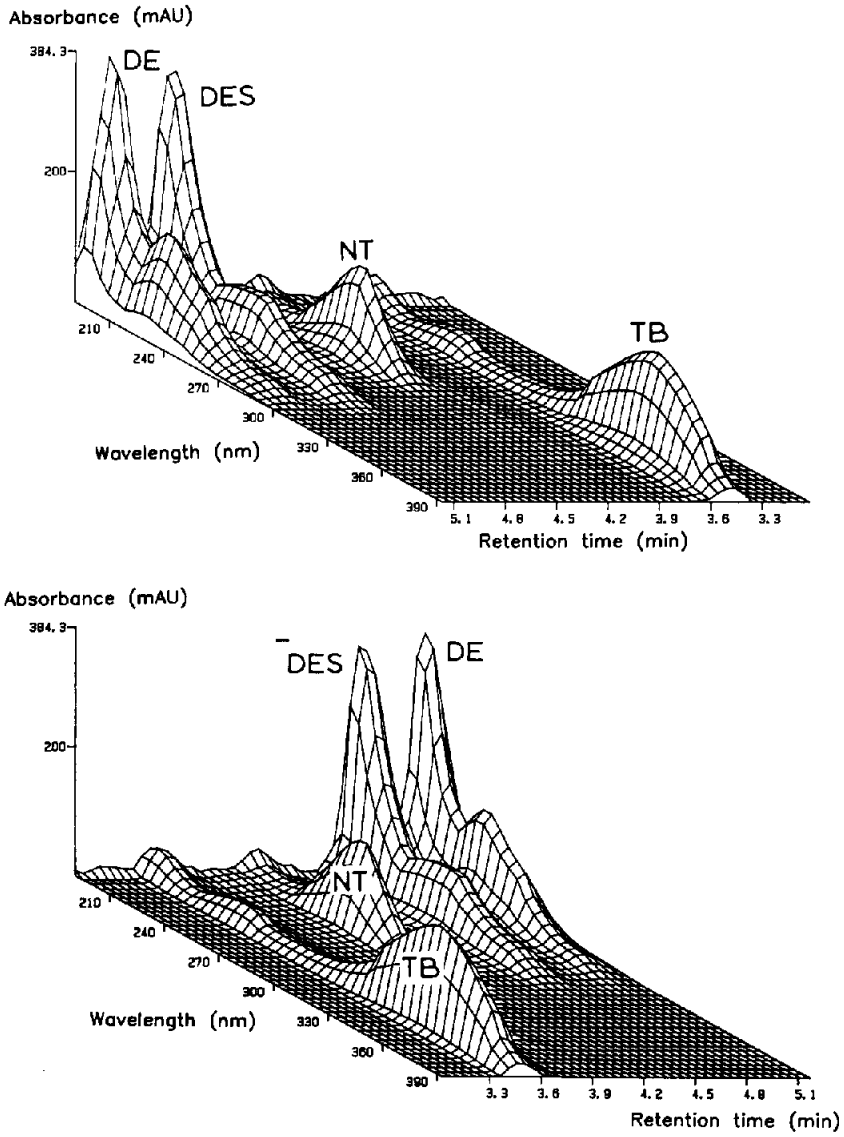

Fig. 5. Three-dimensional plot for a mixture of anabolic standards containing TB, NT, DES and DE from different points of view.

cies were found, the HPLC–diode-array method seems to be a good alternative to GC–MS analysis and, in addition, is faster and cheaper.

A more extensive review has been published by De Beer [3], who reported retention times of 86 anabolic compounds and other steroids (esters) and the use of HPLC with diode-array detection.

In addition to UV and diode-array detection, other on-line detection methods have been reported, such as electrochemical detection [12] and the very interesting detection of enzymatically produced NADH as parameter for the detection of metabolites of anabolic compounds which are not visible in the UV region [13].

3.3. Immunochemical detection

The combination of HPLC separation and off-line immunochemical detection brings both high specificity and sensitivity in the analysis of anabolic compounds in forensic investigations.

3.3.1. HPLC immunogram procedure

A sample extract from urine, faeces, injection

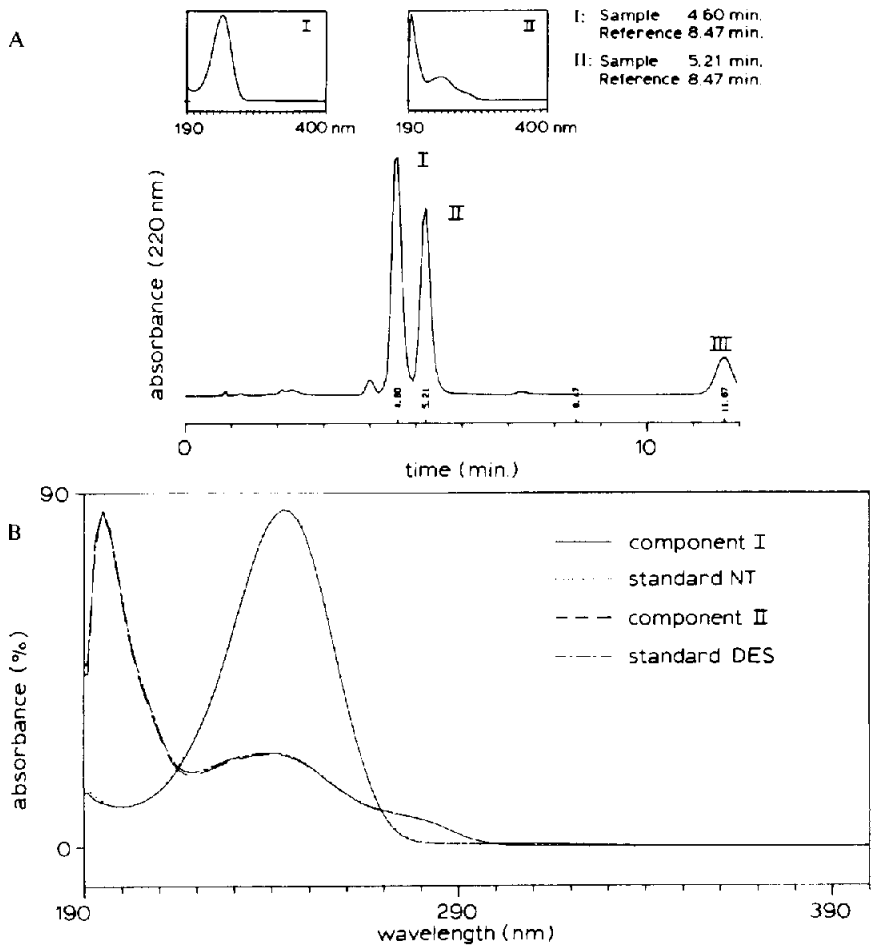

Fig. 6. Example of the identification of NT and DES in a sample from an application site. (A). Chromatogram of an extract from the isolated residue from an application site with the corresponding spectra from 190 to 400 nm of the two components. (B) Identification of components I and II by comparison of the normalized spectra of NT and DES standards, respectively. Reprinted from ref. 11, with permission.

site or other animal tissues is prepared and dissolved in the HPLC mobile phase as a clear solution. After injection into a suitable HPLC system for the separation of the anabolic compounds of interest, the eluate of the complete HPLC run is fractionated into a number of fractions by the use of an on-line controlled fraction collector. The collection time of each fraction can be variable but must be of the order of 10 s. A shorter collection time results in a higher resolution and consequently in more fractions to be analysed. A longer collection time results in the opposite situation. It appeared that a fractionation rate of six times per minute gives a suitable resolution and a number of tubes that can be handled in one immunoassay.

The fractions of the HPLC eluate are evaporated and the residues are dissolved in 1.5 ml of assay buffer. From these fractions, aliquots of 100–200 μl are applied to the immunoassay. The immunoassay can be carried out with different performances as described in the next section. The immunochemical responses of each fraction are plotted against the fraction number or the retention time of the HPLC analysis. With the last method the time delay between the detector

and the fraction collector must be taken into account. Then the retention time(s) of the immunochemical responses are compared with those of a standard mixture of anabolic compounds that have been determined in a separate run using UV detection. If the retention time of an immunochemically responding component is identical (within 0.2 min) with that of a known standard compound and the antiserum used in the immunoassay is directed against this compound, it is concluded that a positive identification has been obtained.

3.3.2. Immunochemical detection techniques

An immunochemical assay for an anabolic compound consists of a specific antiserum raised against the compound of interest and a label.

3.3.2.1. Labels. The kind of label determines the method of detection. Radioimmunoassays (RIA), which use a radioactive label, were developed for a number of anabolic compounds several years ago [14–19]. The last fifteen years have seen a continuous development from the use of radiolabels to non-isotopic alternatives, such as enzymes [20], fluorescent compounds [21], latex particles [22], metal sols [23], free radicals [24], rare earth metals [25] and chemiluminescent compounds [26–28]. In immunoassays for anabolic compounds the most non-isotopic applications have been reported with enzymes [4,29–32] and chemiluminescent labels [33–39]. A comparison between chemiluminescence and spectrophotometric detection with the same enzyme (horseradish peroxidase) showed no increase in the sensitivity in the chemiluminescence immunoassay, as would be expected [31,34]. A review of chemiluminescent immunoassays in veterinary and food analysis has appeared recently [40].

Chemiluminescent labels have been shown to possess a number of advantages [41]. Not only do they have a great intrinsic activity, but also the detection can be performed in a very short period of time and with simple measuring devices. These features make chemuliminescence immunoassays suitable for use in situations where no advanced laboratory facilites are available using, for instance, detection on a sensitive photoplate [42]. In addition, the possibility of simple one-step homogeneous immunoassays can be developed [43–45].

The chemiluminescent labels can be divided into two classes with respect to the kinetics of the chemiluminescence signal. First there are the transient labels, such as luminol and acridinium compounds, which show very fast flash-like kinetics after the initiation of the chemiluminescent reaction. The reaction is started by the injection of hydrogen peroxide. Second, the enzyme labels can be used also in chemiluminescence reactions in which a continuous oxidation reaction of luminol occurs. Examples are horseradish peroxidase [46] and xanthine oxidase [47]. Alkaline phosphatase [48] can also produce a chemiluminescence signal by the generation of a relatively unstable dioxetane. This unstable intermediate decomposes with the production of chemiluminescence. The advantage of all enzyme labels is the production of a long-lasting steady-state signal. The enzyme-enhanced chemiluminescence signals can last for several minutes, hours or even days in the case of xanthine oxidase [49].

The enzyme labels have a number of advantages over the transient labels. Whereas the transient labels require an advanced injection system incorporated in the luminometer, the enzyme labels can be measured very conveniently because of the long duration of the signal. In addition, enzyme labels can supply a greater sensitivity than transient labels, as reported by Van den Berg *et al.* [50]. They claimed a detection limit for NT of 0.04 pg per tube in a solid-phase chemiluminescent immunoassay using an NT–N-(4-aminobutyl)-N-ethylisoluminol (ABEI) label.

As the chemiluminescent labels appeared not to be as stable as expected, the steroid–ABEI labels have to be checked for purity. A procedure for the periodic quality control of ABEI labels has been published by Jansen *et al.* [51]. They used an HPLC separation and fractionation method on a reversed-phase column followed by both off-line immunochemical and chemiluminescence detection. From the results of these two detection methods combined with the results of the HPLC–UV pattern, a conclusion about the

purity of the label preparation can be drawn. An example is shown in Fig. 7 for a testosterone–ABEI label.

3.3.2.2. Antisera. The characteristics of the antiserum used in immunoassays determine both the sensitivity and the specificity. The sensitivity of an immunoassay depends on the combination of antiserum and label. Preferably high antiserum titres are required in order to use higher dilutions of the antiserum, which minimizes the aspecific binding.

The specificity of immunoassays with non-isotopic labels can be adjusted by making use of the site of attachment of the anabolic compound for both the protein moiety of the immunogen and the reporter group of the label. In the so-called homologous immunoassay system, both the label and the immunogen have the same site of attachment. In the heterologous immunoassay system, the sites of attachment of the label and the immunogen are different. Especially with anabolic steroids the specificity can be influenced substantially by using combinations of heterologous assay systems. Examples have been described by Jansen and co-workers for MT [52] and NT [36].

The use of monoclonal antibodies [53] in immunoassays of anabolics has mainly the advantage of having an inexhaustible source of the same quality of antiserum, rather than an increase in specificity [38].

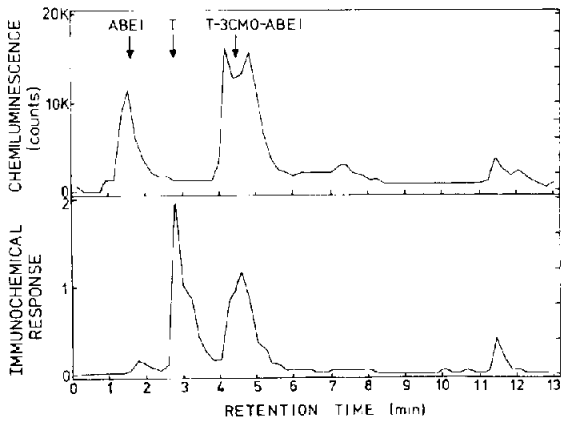

Fig. 7. Quality control of the chemiluminescent testosterone-3-carboxymethyl oxime–ABEI (T-3CMO–ABEI) label. Upper trace: chemiluminescence of the HPLC fractions of the T-3CMO–ABEI preparation. Lower trace: radioimmunochemical response of the same fractions. Retention times of standards are indicated by arrows. Reprinted from ref. 51.

In analytical investigations for the presence of anabolics, either a very high specificity may be required in order to identify unknown compounds in an animal tissue or excreta or a relatively lower specificity for a number of compounds. The last application of a less specific immunoassay can be found in investigations of metabolism or in multi-component immunoassays [7].

3.3.3. Applications

The immunogram procedure was applied for the first time by Jansen *et al.* [54] in the identification of the stilbenes DES, DE and HEX in bovine urine samples (Fig. 8).

In addition to the positive identification of known stilbenes, the method was also able to detect unknown responses which in a normal immunoassay would lead to unwanted false-positive results. The immunochemical method used was RIA. A similar procedure was reported [55] for the stilbenes but with chemiluminescence immunochemical detection using the chemiluminogenic ABEI coupled to HEX. Very low detection limits were obtained in this system of 10 pg of HEX per tube (at 90% relative binding).

The immunogram procedure for NT was reported by Jansen *et al.* [37] in the identification of this exogenous androgen in both bovine urine and the application site. An immunochemical procedure with chemiluminescence detection was used. Other reports concerning the presence of NT in bovine urine [7], kidney fat [4], milk replacers [39] and bovine muscle tissues [56] described the usefulness of the HPLC immunogram procedure. Rapp and Meyer [39] were also able to detect the presence of 17α-nortestosterone and 19-norandrostenedione using an enzyme immunoassay.

Identifications of the synthetic androgen MT by HPLC immunogram procedures have been reported by Jansen and co-worker [7,35,36] and Daeseleire *et al.* [56]. Applications have been described both for urinary MT and its presence in application sites [7,35,36] and in bovine meat

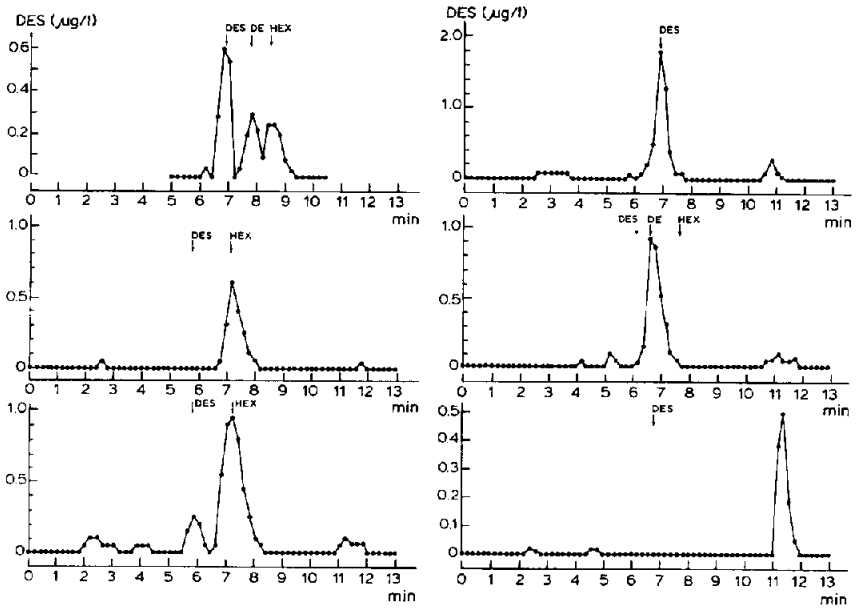

Fig. 8. HPLC immunograms with radioimmunochemical detection using RIA for DES for six different samples of bovine urine. The retention times of stilbene standards are indicated by arrows. Reprinted from ref. 54, with permission.

samples [56]. Another application has been described in which the HPLC immunogram procedure was applied to urine from a veal calf injected with MT in a model experiment [52]. In addition to the quantitative excretion of the parent compound, the presence of at least one other metabolite, which was not identified, was observed.

Jansen *et al.* [38] described the application of an HPLC immunogram procedure for zeranol in a metabolic study of the fate of zeranol in male veal calves. In a model experiment the presence and different excretion profiles of three components in urine could be detected quantitatively (Fig. 9). Because of the broad specificity of the antiserum used, not only was the parent compound zeranol detected but also its epimer taleranol and metabolite zearalanone.

Another example of HPLC immunograms, which has not been published, is the identification of trenbolone metabolites in urine samples from veal calves. Fig. 10 shows an example in which the α- and β-isomers of trenbolone were identified in bovine urine. The arrows indicate the retention times of standard compounds.

Rapp and Meyer [39] applied HPLC immunogram procedures to E2 benzoate and MP acetate.

Another interesting development is the off-line detection of HPLC fractions with radioreceptor assays, as described for estrogenic compounds in bovine urine [57].

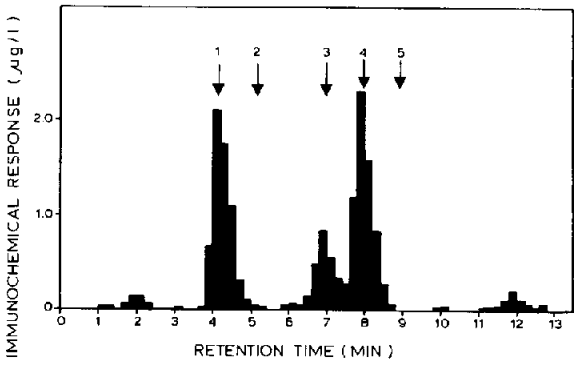

Fig. 9. HPLC immunogram of an extract of urine from a calf three days after treatment with zeranol using detection via a chemiluminescent immunoassay for zeranol. The retention times of zeranol standards are indicated by arrows. Reprinted from ref. 7.

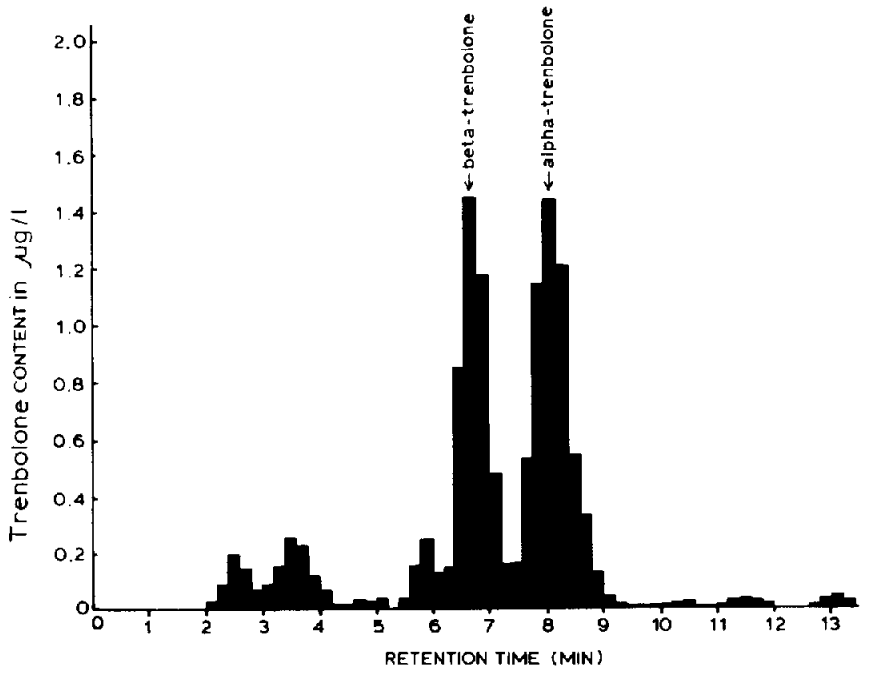

Fig. 10. HPLC immunogram of an extract of bovine urine using detection via RIA for trenbolone. The retention times of standards are indicated by arrows.

4. CONCLUSIONS

HPLC has proved to be an excellent separation method for anabolic compounds. In addition to reversed-phase separations on C_{18} columns, normal-phase columns can also be used. The separations can be used for both on- and off-line detection and for preparative purification of the particular compounds from a biological matrix.

A number of detection methods can be used, each of which has its own characteristics and advantages. The simplest detection method is UV–VIS detection at a single wavelength. The development of very sensitive UV detectors allows detection at the nanogram level. The introduction of multi-wavelength detection and diode-array detection has improved the specificity substantially. In combination with the use of advanced computerized spectral comparisons, the identification of anabolic compounds can be performed in an unambiguous way. If both specificity and sensitivity of detection of anabolic compounds in complex biological matrices are required, the use of off-line immunochemical detection can be very helpful. Also the detection and identification of metabolites, which are often required in toxicological evaluations [58], can be done with the immunogram technique if the specificity of the antisera allows metabolite detection. Because of the development of very sensitive non-isotopic detection methods, the use of radioactivity is no longer a barrier for the use of this technique. Limits of detection in the picogram range can be achieved with the use of enzyme or chemiluminescent labels.

From the foregoing, it will be clear that the use of HPLC in the analysis and detection of anabolic compounds is a strict requirement for good and reliable results.

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REFERENCES

1 E. H. J. M. Jansen and R. W. Stephany, *Vet. Q.*, 7 (1985) 35.
2 E. H. J. M. Jansen, R. Both-Miedema, H. van Blitterswijk and R. W. Stephany, *J. Chromatogr.*, 299 (1984) 450.
3 J. O. De Beer, *J. Chromatogr.*, 489 (1989) 139.
4 H. H. D. Meyer, F. X. Hartman and M. Rapp, *J. Chromatogr.*, 489 (1989) 173.
5 E. H. J. M. Jansen, R. Both-Miedema and R. H. van den Berg, *J. Chromatogr.*, 489 (1989) 57.
6 C. Gertz and W. Fellmann, *Fresenius' Z. Anal. Chem.*, 323 (1986) 343.
7 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.
8 E. H. J. M. Jansen, H. van Blitterswijk, P. W. Zoontjes, R. Both-Miedema and R. W. Stephany, *J. Chromatogr.*, 347 (1985) 375.
9 E. H. J. M. Jansen, P. W. Zoontjes, R. Both-Miedema, H. van Blitterswijk and R. W. Stephany, *J. Chromatogr.*, 347 (1985) 379.
10 E. H. J. M. Jansen, P. W. Zoontjes, H. van Blitterswijk, R. Both-Miedema and R. W. Stephany, *J. Chromatogr.*, 319 (1985) 436.
11 E. H. J. M. Jansen, H. van Blitterswijk and R. W. Stephany, *Vet. Q.*, 6 (1984) 60.
12 T. Reuvers, E. Perogordo and R. Jimenez, *J. Chromatogr.*, 564 (1991) 477.
13 J. P. Cravedi and G. Delous, *J. Chromatogr.*, 564 (1991) 461.
14 S. N. Dixon, *J. Vet. Pharmacol. Ther.*, 3 (1980) 177.
15 T. J. Benraad, R. W. Stephany, F. M. A. Rosmalen, J. A. Hoffmann, J. G. Loeber and L. H. Elvers, *Vet. Q.*, 3 (1981) 866.
16 J. P. Duchatel and G. G. Maghuin-Rogister, *Ann. Med. Vet.*, (1983) 114.
17 E. H. J. M. Jansen, R. H. van den Berg, G. Zomer and R. W. Stephany, *J. Clin. Chem. Clin. Biochem.*, 23 (1985) 145.
18 E. H. J. M. Jansen, R. H. van den Berg, H. van Blitterswijk, R. Both-Miedema and R. W. Stephany, *Food Addit. Contam.*, 2 (1985) 271.
19 B. Hoffmann and G. Oetell, *Steroids*, 27 (1976) 509.
20 A. H. W. M. Schuurs and B. K. van Weemen, *Clin. Chim. Acta*, 81 (1977) 1.
21 E. Sioni and I. Hemmilae, *Clin. Chem.*, 25 (1979) 353.
22 C. L. Cambiaso, A. E. Leek, F. de Steenwinkel, J. Billen and P. L. Masson, *J. Immunol. Methods*, 18 (1977) 33.
23 J. W. H. Leuvering, P. J. H. M. Thal, M. van der Waart and A. H. W. M. Schuurs, *Fresenius' Z. Anal. Chem.*, 301 (1980) 132.
24 R. Wei and R. Almirez, *Biochem. Biophys. Res. Commun.*, 62 (1975) 510.
25 T. N. Lovgren, *J. Steroid Biochem.*, 27 (1987) 47.
26 H. R. Schroeder, R. C. Bogulaski, R. J. Carrico and T. Buckler, *Methods Enzymol.*, 57 (1978) 424.
27 T. P. Whitehead, G. H. G. Thorpe, T. J. M. Carter, C. Groucutt and L. J. Kricka, *Nature (London)*, 305 (1983) 158.
28 I. Weeks, I. Beheshti, F. McCapra, A. K. Campbell and J. S. Woodhead, *Clin. Chem.*, 29 (1983) 1471.
29 G. Degand, P. Schmitz and G. Maghuin-Rogister, *J. Chromatogr.*, 489 (1989) 235.
30 E. H. J. M. Jansen, R. H. van den Berg and G. Zomer, in K. Van Dyke and R. Van Dyke (Editors), *Luminescence Immunoassay and Molecular Applications*, CRC Press, Boca Raton, FL, 1990, p. 57.
31 E. H. J. M. Jansen, J. J. Bergman, R. H. van den Berg and G. Zomer, *Anal. Chim. Acta*, 227 (1989) 109.
32 J. L. Van Look, E. H. J. M. Jansen, R. H. van den Berg, G. Zomer, K. E. Vanoosthuyze and C. H. Van Peteghem, *J. Chromatogr.*, 564 (1991) 451.
33 C. Van Petheghem, L. Van Look and A. De Guesquiere, *J. Chromatogr.*, 489 (1989) 219.
34 L. J. Van Look, E. H. J. M. Jansen, R. H. van den Berg and C. H. Van Peteghem, in P. E. Stanley and L. J. Kricka (Editors), *Bioluminescence and Chemiluminescence. Current Status*, Wiley, New York, 1991, p. 159.
35 E. H. J. M. Jansen, R. H. van den Berg, G. Zomer and R. W. Stephany, *Food Addit. Contam.*, 2 (1985) 47.
36 E. H. J. M. Jansen, C. A. Laan, R. H. van den Berg and R. W. Stephany, *Anal. Chim. Acta*, 170 (1985) 29.
37 E. H. J. M. Jansen, G. Zomer, R. H. van den Berg and R. W. Stephany, *Vet. Q.*, 6 (1984) 101.
38 E. H. J. M. Jansen, R. H. van Berg, G. Zomer, C. Enkelaar-Willemsen and R. W. Stephany, *J. Vet. Pharmacol. Ther.*, 9 (1986) 101.
39 M. Rapp and H. H. D. Meyer, *J. Chromatogr.*, 489 (1989) 181.
40 E. H. J. M. Jansen, G. Zomer and C. H. Van Peteghem, in W. R. G. Baeyens, D. De Keukeleire and K. Korkidis (Editors), *Luminescence Techniques in Chemical and Biochemical Analysis*, Marcel Dekker, New York, 1991, p. 477.
41 W. G. Wood, *J. Clin. Chem. Clin. Biochem.*, 22 (1984) 905.
42 E. H. J. M. Jansen, C. A. F. Buskens and R. H. van den Berg, *J. Chromatogr.*, 489 (1989) 245.
43 M. Pazzagli, G. F. Bolleli, G. Messeri, G. Martinazzo, A. Tomassi, R., Salerno and M. Serio, in M. Serio and M. Pazzagli (Editors), *Luminescent Assays: Perspectives in Endocrinology and Clinical Chemistry*, Raven, New York, 1982, p. 191.
44 G. Messeri, A. L. Caldini, G. F. Bolelli, M. Pazzagli, A. Tomasi, P. L. Vannuchi and M. Serio, *Clin. Chem.*, 30 (1984) 653.
45 G. Zomer, R. H. van den Berg and E. H. J. M. Jansen, in J. Scholmerich, R. Andreesen, A. Kapp, M. Ernst and W. G. Wood, (Editors), *Bioluminescence and Chemiluminescence. New Perspectives*, Wiley, New York, 1987, p. 305.

46 G. H. G. Thorpe, L. J. Kricka, E. Gillespie, S. Moseley, R. Amess, N. Baggett and T. P. Whitehead, *Anal. Biochem.*, 145 (1985) 96.
47 A. Baret and V. Fert, *J. Biolumin. Chemilumin.*, 4 (1989) 149.
48 I. Bronstein, B. Edwards and J. C. Voyta, *J. Biolumin. Chemilumin.*, 4 (1989) 99.
49 E. H. J. M. Jansen, R. H. van den Berg and G. Zomer, *J. Biolumin. Chemilumin.*, 4 (1989) 129.
50 R. H. van den Berg, E. H. J. M. Jansen, G. Zomer, C. Enkelaar-Willemsen, C. A. Laan and R. W. Stephany, *Anal. Chim. Acta*, 205 (1988) 243.
51 E. H. J. M. Jansen, R. H. van den Berg, R. Both-Miedema, C. Enkelaar-Willemsen and G. Zomer, *Anal. Chim. Acta*, 205 (1988) 175.
52 E. H. J. M. Jansen, R. H. van den Berg, R. Both-Miedema, C. Enkelaar-Willemsen, G. Zomer and R. W. Stephany, in J. Scholmerich, R. Andreesen, A. Kapp, M. Ernst and W. G. Wood (Editors), *Bioluminescence and Chemiluminescence. New Perspectives*, Wiley, New York, 1987, p. 265.
53 A. P. Carter, S. N. Dixon and M. H. Bew, *J. Vet. Pharmacol. Ther.*, 7 (1984) 17.
54 E. H. J. M. Jansen, R. H. van den Berg, H. van Blitterswijk, R. Both-Miedema and R. W. Stephany, *Vet. Q.*, 6 (1984) 5.
55 R. H. van den Berg, E. H. J. M. Jansen, G. Zomer, C. Enkelaar-Willemsen, R. Both-Miedema and R. W. Stephany, *J. Chromatogr.*, 360 (1986) 449.
56 E. Daeseleire, A. De Guesquiere and C. Van Peteghem, *J. Chromatogr.*, 564 (1991) 445.
57 C. J. M. Arts and H. van den Berg, *J. Chromatogr.*, 489 (1989) 225.
58 M. Metzler, *J. Chromatogr.*, 489 (1989) 11.