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METABOLISM OF SOME ANABOLIC AGENTS: TOXICOLOGICAL AND ANALYTICAL ASPECTS

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SUMMARY

The metabolism of the animal growth promotants diethylstilbestrol, zeranol and 17β-trenbolone and of a few anabolizing steroids used in humans is briefly reviewed. The possible role of reactive metabolic intermediates in the toxicity of some anabolic agents is discussed. Analytical implications of the metabolism of anabolizing agents are described and examples of the analysis of metabolites by means of recently developed techniques are given. It is proposed to utilize the covalent binding of reactive metabolites of anabolic compounds to blood proteins such as haemoglobin and serum albumin for retrospective doping analysis.

INTRODUCTION

Anabolizing agents are compounds that stimulate protein synthesis and thus increase muscle size and strength both in humans and in animals. In addition to their medical applications, anabolic agents are used by athletes in order to improve their performance in competitive sports. For the same purpose they are given to animals participating in sporting events, e.g., to racehorses. In food-producing animals, anabolics increase the slaughter weight and improve the food conversion rate. Those compounds effective in humans and horses are invariably steroids structurally related to the natural androgen testosterone, whereas in cattle androgenic and estrogenic agents or combinations of both may be used. The estrogens may be derived from the natural steroid 17β-estradiol or may have a non-steroid structure, e.g. diethylstilbestrol or zeranol.

The non-medical use of anabolic agents in athletes has raised mainly ethical questions. However, as side-effects of these compounds have been described, which may range from hormonal disturbances to neoplasia after prolonged use, there is increasing concern about the toxicology involved. For ethical and health reasons,

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the use of anabolics prior to and during contests is generally forbidden by most sporting organizations. In food-producing animals, the risk posed by residues of the anabolic to the health of the consumer is the major concern. At present, certain anabolics can be legally given to farm animals in some countries but are banned in others.

The necessity to test for illegal use or to determine residue levels after legal use has led to a strong interest in methods for the detection of anabolizing agents in biological samples. Interest in the toxicological effects of anabolics in food is also growing, owing to an increased sensitivity of the public towards environmental exposure to chemicals. However, because very little is known about the mechanisms underlying the toxic effects of anabolics, the assessment of the risks associated with these agents is a very difficult task at present.

For the analysis of biological samples and for an understanding of the toxicity of a compound, pharmacokinetic and metabolic considerations are of the utmost importance. This paper briefly reviews recent data on the metabolism of some selected anabolic agents, emphasizing toxicological and analytical aspects.

DIETHYLSTILBESTROL

The synthetic estrogen diethylstilbestrol (DES) was legally used in the U.S.A. as growth promoter in beef cattle until it was banned by the U.S. Food and Drug Administration in 1979. Episodes of illegal use have occurred in several countries over the years.

DES is carcinogenic in several animal species and is considered to be a transplacental carcinogen in humans [1]. Therefore, it has attracted attention as a model compound for studies of the mechanisms of hormonally active carcinogens. Studies in several animal models suggest that oxidative metabolites of DES play a role in the mechanism of DES-induced cancer.

The oxidative metabolism of radioactively labelled DES has been studied in vivo in several species, including man, and also in various in vitro systems [2,3]. A summary of the metabolic pathways is depicted in Fig. 1.

For the separation of the various oxidative DES metabolites, both gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used [4,5]. With either method, the *E*- and *Z*-isomers of each metabolite show up in the chromatogram because of the partial isomerization that occurs when DES and its metabolites are exposed to daylight.

Several reactive metabolites are formed in DES metabolism, e.g., arene oxides as precursors of the catechols and semiquinone- and quinone-type metabolites derived by one-electron oxidation of DES and 3′-hydroxy-DES. However, the significance of these metabolites for the carcinogenicity of DES is still under debate as they do not bind to DNA. Studies in Syrian hamster embryo fibroblasts, which are neoplastically transformed by DES in vitro, suggest that induction of near-diploid aneuploidy may be the first step in DES-induced neoplastic cell transformation [6]. We have recently reported that DES-4′,4″-quinone (Fig. 1) binds in vitro covalently and fairly specifically to β-tubulin, a major component of the spindle apparatus [7]. This binding of a reactive DES metabolite might

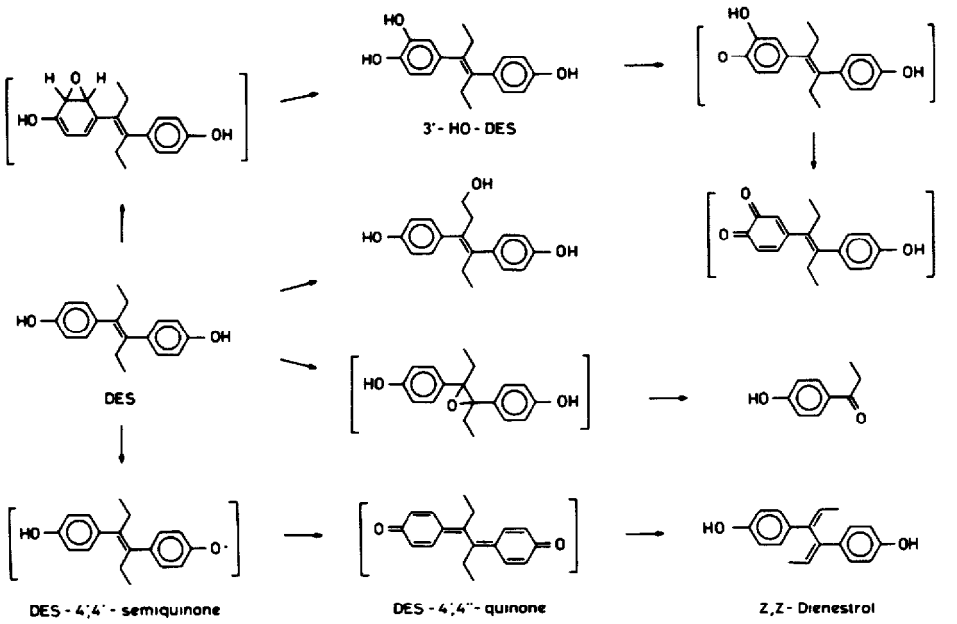

Fig. 1. Major pathways in the metabolism of diethylstilbestrol (DES). Only the *trans* isomers are shown. Compounds in brackets denote putative reactive intermediates.

represent the biochemical mechanism of aneuploidy induction. According to Barrett et al. [8], the biological sequelae of DES-induced aneuploidy could be changes in cellular oncogenes and suppressor genes which might eventually lead to the neoplastic state.

The proposed mechanism is of particular interest because it provides a basis for understanding the induction of genetic damage (aneuploidy) by interaction of the carcinogen with certain proteins in the absence of DNA damage. This has a considerable bearing on the risk assessment for hormonal carcinogens, and it should prove interesting to test whether other anabolic agents can also act as aneuploidogens.

ZERANOL

Zeranol (7α-zeralanol) is a synthetic derivative of the mycoestrogen zearalenone, which occurs in *Fusarium* moulds. Zeranol is a weak estrogen and is currently used as a growth promoter in livestock in the U.S.A. The metabolism of tritium-labelled zeranol has been studied in the rat, rabbit, dog, monkey and man [9] and the major oxidative pathways are depicted in Fig. 2.

Column and thin-layer chromatography were used for the separation of zeranol metabolites in the above-mentioned study [9]. More recently, reversed-phase HPLC has been demonstrated to separate zeranol, taleranol and zearalanone effectively [10]. By using capillary gas chromatography–mass spectrometry (GC–MS), Covey et al. [11] were able to resolve these three compounds and to sepa-

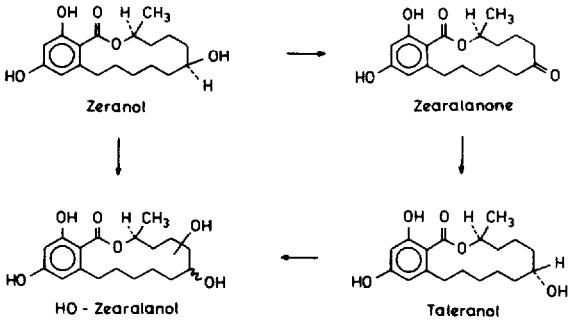

Fig. 2. Metabolic pathways of zeranol.

TABLE I

SPECIES DIFFERENCES IN THE URINARY METABOLISM OF ZERANOL

Data from ref. 9.

Species	Dose in urine (%)	Zeranol (% of dose)		Zearalenone (% of dose)		Unknown (% of dose)
		Free	Conjugated	Free	Conjugated	
Man	55.1	1	19.4	1	12.8	22.9
Monkey	20.5	9.8	1.9	3.6	0.3	5.0
Dog	3.8	0.5	0.6	0.1	0.1	1.5
Rabbit	75.2	1.6	16.4	13.6	7.8	21.2
Rat	9.2	0.5	1.9	1.1	3.1	2.7

rate them from various other estrogens such as DES, (*E,E*)-dienestrol, *meso*-hexestrol, zearalenone, α- and β-zearalenol, 17β-estradiol and estriol.

A quantitative survey of the urinary metabolites of zeranol (Table I) reveals pronounced species differences in the route of excretion, the amounts of free and conjugated metabolites and the extent of oxidation. Moreover, in some species a considerable proportion of the metabolites still awaits structural identification. No evidence for the formation of reactive metabolites has been reported so far.

TRENBOLONE

17β-Trenbolone (17β-TBOH) is a synthetic steroidal androgen. Its acetate (TBA) is legalized for growth promotion of beef cattle in the U.S.A. and a few European countries. Biotransformation studies have been carried out in rats and cows by analysing the biliary metabolites after a single dose of TBA labelled with tritium in the steroid ring [12]. About 80% of the dose was recovered in the 24-h bile in both species and a number of oxidative metabolites were identified (Fig. 3). In both species, TBA was rapidly hydrolysed. However, a marked difference in the amounts of the various metabolites was noted between rats and cows (Table II). 17β-TBOH and the 17-keto compound triendione together with their 16α- and 16β-hydroxylated products predominated in the rat. In heifer bile, these

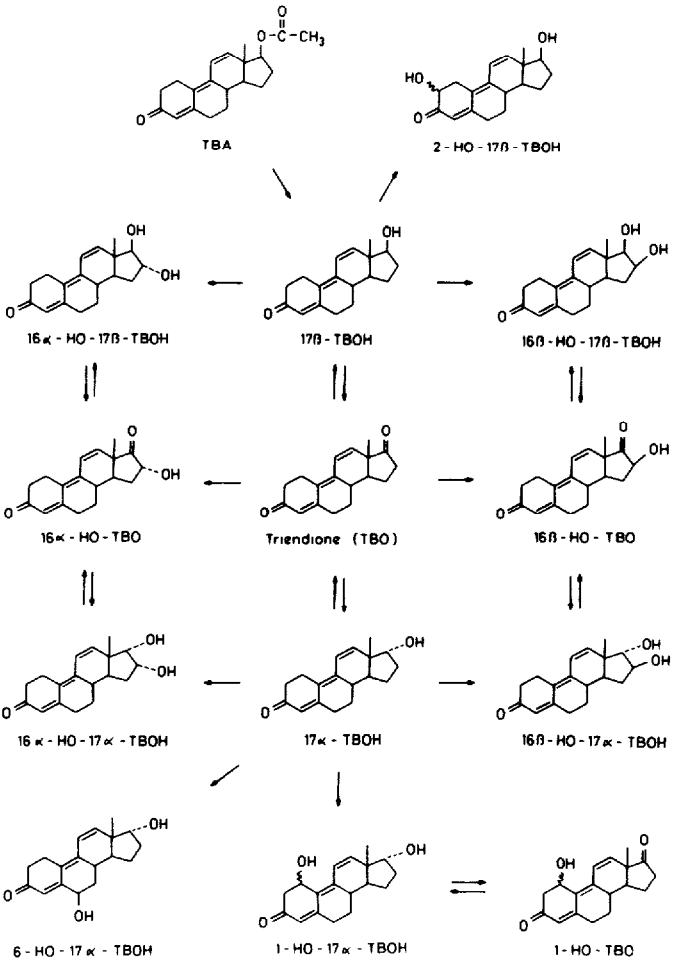

Fig. 3. Metabolism of 17β-trenbolone (17β-TBOH).

metabolites were negligible and 17-epi-TBOH (17α-TBOH) was the major product together with small amounts of 16α- and 16β-hydroxy-17α-TBOH.

Chromatographic methods employed for the separation of these metabolites were mostly based on silica gel adsorption chromatography carried out in columns or on thin-layer plates [12]. More recent studies aimed at the determination of β-TBOH and its major metabolite 17α-TBOH in bovine liver and muscle have used reversed-phase HPLC with on-line tandam mass spectrometry [13] and capillary GC–MS [14]. However, a chromatographic system for the separation of all the metabolites of 17β-TBOH in one run has not yet been established.

When the residues in bovine liver and muscular tissue were analysed after implantation of radiolabelled TBA, it was found that only 5–15% of the radioactivity was extractable with an organic solvent [15]. About half of the remaining radioactivity was extractable with water and the other half could be liberated by treatment with proteolytic enzymes. This suggests a covalent binding of 17β-TBOH or its metabolite(s) to protein. A small but definite covalent protein bind-

TABLE II

COMPARISON OF THE BILIARY METABOLITES OF 17β-TRENBOLONE ACETATE IN RAT AND COW

Data from ref. 12.

Metabolite	Percentage of excreted radioactivity	
	Rat	Heifer
17β-TBOH acetate (TBA)	–	–
17β-TBOH	20.6	0.9
2-Hydroxy-17β-TBOH	0.6	–
16α-Hydroxy-17β-TBOH	10.5	0.7
16β-Hydroxy-17β-TBOH	3.4	–
Triendione	2.4	1.2
16α-Hydroxy-triendione	17.1	1.3
16β-Hydroxy-triendione	1.5	–
1-Hydroxy-triendione	1.8	0.2
Epi-trenbolone (17α-TBOH)	–	33.8
1-Hydroxy-17α-TBOH	–	0.2
16α-Hydroxy-17α-TBOH	–	3.0
16β-Hydroxy-17α-TBOH	–	3.0
6β-Hydroxy-17α-TBOH	–	0.4

ing was also observed when [^{3}H]17β-TBOH was incubated with a calf liver homogenate in vitro [15]. The toxicological significance of the protein binding of 17β-TBOH is as yet unclear. Although extensive studies in various short-term assays have revealed that 17β-TBOH has very little if any DNA-damaging potential [16], we have recently reported that both 17β-TBOH and 17α-TBOH are able to transform Syrian Hamster embryo cells neoplastically in vitro and to induce the formation of micronuclei in the same cells [17,18]. It should prove interesting to clarify whether 17β-TBOH can act as a chromosomal mutagen by inducing aneuploidy in these and in other cells.

OTHER ANABOLIC STEROIDS

In human medicine and athlete doping, a number of steroids structurally related to the natural androgen testosterone are used. The structures of some of these compounds are depicted in Fig. 4.

The separation and unequivocal identification of the various anabolic steroids is of paramount importance in doping-control analyses. GC–MS on capillary columns with selected-ion monitoring is currently used for this purpose. A detailed account of the procedure is beyond the scope of this review; the reader is referred to two recent papers on this subject [19,20].

Regrettably, data on the metabolism of anabolic steroids in laboratory animals and in humans are scarce and not readily available in the accessible literature. No systematic studies with radiolabelled compounds have apparently been car-

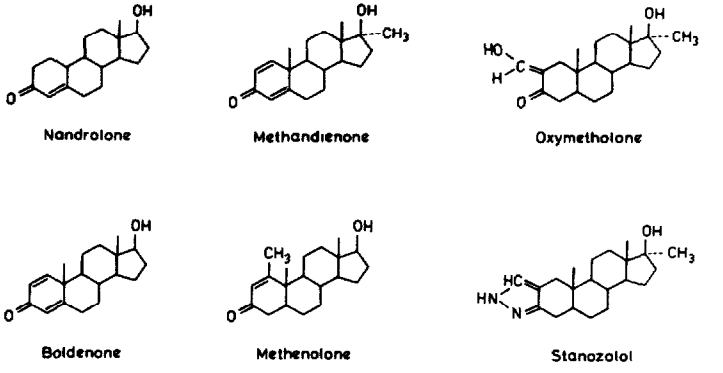

Fig. 4. Structures of some anabolic steroids.

ried out to clarify the pharmacokinetic and metabolic profiles of these widely used substances. One of the few exceptions concerns 19-nortestosterone (nandrolone) and 1-dehydrotestosterone (boldenone). Their fate has been studied in racehorses with tritium-labelled steroids in Houghton's laboratory [21–23]. These studies and a few others with unlabelled steroids in humans, e.g., of boldenone [24], methandienone [25–27] and oxymetholone [26,27], seem to indicate that the metabolism of the synthetic steroids differs significantly from that of testosterone. Testosterone is efficiently reduced at the 4,5-double bond and at the 3-keto group by the enzymes 5α-reductase and 3α/β-hydroxysteroid oxidoreductase, respectively, and oxidized at the 17β-hydroxy group by 17-hydroxysteroid dehydrogenase. Introduction of additional double bonds, alkylation at position 17 or removal of the C-19 methyl group, hinders these metabolic reactions. As a consequence, metabolism of the A ring decreases and at other sites of the molecule reactions such as hydroxylation in positions 6 and 16 occur. Comparative studies of ^{3}H-labelled testosterone and nandrolone in various tissues of the rat in vivo and in vitro support this supposition [28], as do the metabolic data for 17β-TBOH discussed earlier.

Whether this shift in metabolism has toxicological significance is as yet unknown. Apparently none of the synthetic steroidal anabolics used in humans or their metabolites have ever been tested in short-term assays, in spite of the fact that there are case reports associating the use of these compounds with liver cancer in humans [29]. Potentially, 16-hydroxylation could be a metabolic activation reaction, as 16α-hydroxyestrone has been demonstrated recently to bind covalently to proteins [30]. The formation of this metabolite has been causally implicated in estradiol-induced breast cancer in humans [31]. Metabolites containing hydroxy groups in conjugation with olefin double bonds or keto groups might also be of toxicological interest, as esters of allylic alcohols have electrophilic reactivity [32]. Olefinic double bonds could also give rise to reactive epoxide intermediates.

In view of the potential of anabolic steroids for metabolic activation, the metabolism of these compounds should be systematically studied using radiotracer techniques and the agents and their metabolites should be tested in short-term assays for induction of genetic damage.

ANALYTICAL IMPLICATIONS OF METABOLISM

For the unequivocal detection of an anabolizing agent in biological samples it may sometimes be of advantage to take the metabolism into account. Typical situations are as follows:

(a) Metabolites may be present in larger amounts than the parent compound, allowing an increase in sensitivity. An example is the screening for trenbolone in cattle. As discussed above, TBA is readily hydrolysed and converted into 17α-TBOH in the bovine liver. Accordingly, only a minute peak of 17β-TBOH but a large peak of 17α-TBOH were observed on the HPLC trace of a liver extract from a cow implanted with TBA [13].

(b) Metabolites may be more easily accessible and/or more suitable for analysis than the parent compound. For example, Weidolf et al. [33] have recently determined boldenone sulpho-conjugate and related steroid sulphates with high sensitivity in equine urine by HPLC–tandem MS. This rendered the enzymatic hydrolysis of the conjugate, which is sometimes incomplete for sulphates, unnecessary. Thus the sample work-up procedure was accelerated and losses were avoided. The increasing use of thermospray interfaces in HPLC–MS can be expected to facilitate the MS analysis of conjugated metabolites in the future.

(c) Metabolites determined in addition to the parent compound facilitate the interpretation and increase the reliability of analysis. For example, bovine liver samples from animals treated with zeranol and from untreated animals were analysed by GC–MS [11]. In the former samples (zeranol administered to animal), both zeranol and its major metabolite, taleranol (see above), were found. Unexpectedly, a large proportion of the liver samples from untreated animals contained similar peaks, which were identified as zearalenone and its major metabolites α- and β-zearalenol. The presence of these compounds was attributed to the use of *Fusarium*-contaminated feed [11].

(d) Metabolites may allow the detection of the exogenous administration of an anabolic compound which is also formed endogenously. An example of this situation was studied by Houghton et al. [23] for 19-nortestosterone. This compound is formed endogenously in untreated colts, as are 3β,17β-estranediol and 3β,17β-estrenediol. Estranediol but not estrenediol is a metabolite of 19-nortestosterone. Therefore, administration of nortestosterone (nandrolone) leads to an increase in the ratio of urinary estranediol to estrenediol, the measurement of which can be effectively used to detect exogenous nortestosterone in colts.

In addition to these established advantages of metabolites in the analysis of anabolizing agents, another application appears feasible, which may prove useful in the future for the retrospective detection of anabolic steroids. However, it should be stressed that this represents a hypothetical approach for which no experience exists so far.

PROPOSAL FOR THE RETROSPECTIVE ANALYSIS OF ANABOLIZING STEROIDS

Among the various classes of doping agents used in athletes, anabolizing steroids take the largest share. For example, doping agents were detected in 623 out

of 32 982 samples analysed in 1986 by 18 laboratories accredited by the International Olympic Committee [19]. Of the 623 positive samples, 439 contained anabolic steroids; stimulants ranked second with 177. The actual use of anabolic steroids in athletes must be expected to be much much higher, as rapidly acting steroids taken prior to the contest are likely to have escaped the doping analysis. There are several possible strategies to detect anabolic steroids used prior to competition: (A) 'Out-of-competition' controls; (B) Retrospective analysis; (1) increased sensitivity for the anabolic steroid; (2) analysis of metabolites with long half-lives; and (3) analysis of covalent adducts of the anabolic steroid or its metabolite(s) with macromolecules, e.g., proteins. In addition to out-of-competition controls and instrumental improvements leading to higher sensitivity, two approaches are conceivable which take into account the metabolites of anabolic agents: (a) the detection of metabolites with a long half-life and (b) the detection of reactive metabolites by means of their reaction products with macromolecules. Both possibilities are as yet hypothetical. To exploit long-lived metabolites for retrospective analysis requires a detailed knowledge of the nature of the metabolites and their pharmacokinetic behaviour. This can only be obtained by radiotracer studies which, as discussed earlier, have yet to be performed. However, as metabolites are, in general, more readily excreted than the parent compounds, it is questionable whether such long-lived metabolites will be found.

The second approach, i.e., the measurement of the reaction products of anabolic agents with macromolecules, requires the formation of reactive metabolites. This has been demonstrated so far for DES and trenbolone (see above) but has apparently not been studied for other anabolizing compounds. However, metabolic considerations for anabolic steroids outlined above make the formation of at least small amounts of reactive intermediates, which should be capable of binding covalently to proteins, seem fairly likely. Covalent DNA binding is less likely in view of the experience with DES and trenbolone. If protein binding with reactive metabolites of anabolic steroids occurs, principles established in recent years for measuring the exposure of individuals to toxic and carcinogenic agents at the workplace can be utilized. These studies of biomonitoring industrial compounds [34] have shown that covalently bound adducts of xenobiotic substances such as aniline, 4-aminobiphenyl, ethylene oxide or propylene oxide to haemoglobin can be detected with high sensitivity (about 10 pmol/g of protein). Owing to the long lifetime of haemoglobin (about 18 weeks in human erythrocytes), repeated doses of the xenobiotic lead to an accumulation of the adduct and measurements can be carried out after prolonged periods of time. The analysis of the adduct can be done after chemical or enzymatic protein degradation by chromatographic (HPLC, GC), spectrometric (MS, UV) or immunological methods (radioimmunoassay). In addition to haemoglobin, serum albumin has been exploited as an easily accessible protein for biomonitoring purposes. Although albumin has a shorter half-life (about 20 days) in man than haemoglobin, it has the potential advantage of being formed in the liver, which is also a major site of xenobiotic metabolism. Short-lived reactive metabolites generated in the liver may therefore be more efficiently trapped by albumin than by haemoglobin.

The concept of monitoring covalent protein adducts of anabolic steroids in

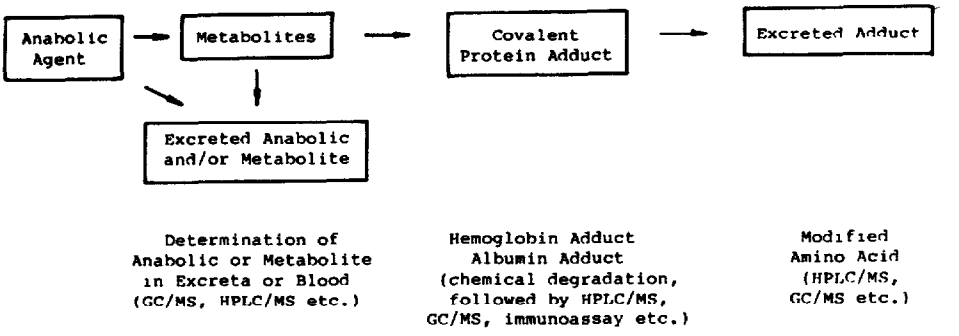

Fig. 5. Integrated concept for the biomonitoring of anabolic steroids.

blood samples or even their biological degradation products (modified amino acids) in urine is shown together with the conventional screening of the anabolics or their metabolites in Fig. 5. It should be emphasized again that the feasibility of the determination of protein adducts of anabolic agents has yet to be demonstrated. This should be done first in animal experiments using radiolabelled anabolics. In view of the promising results in the biological monitoring of toxic agents described above and because of the urgent need to develop sensitive methods for retrospective doping analysis, studies along these lines should be encouraged.

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