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RADIOIMMUNOASSAY OF THE ANTI-CANCER AGENT 4-HYDROXYANDROSTENEDIONE IN BODY FLUIDS

J. KHUBIEH, G. W. AHERNE and J. CHAKRABORTY*
Department of Biochemistry, University of Surrey, Guildford, Surrey GU2 5XH, England

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Summary—Antibodies were produced in sheep against a new anti-breast cancer drug 4-hydroxyandrostenedione (4-OHA) using two hapten-ovalbumin conjugates. One of these conjugates (4-hydroxytestosterone-ovalbumin) produced an antiserum suitable for the development of a radioimmunoassay that would allow direct measurement of 4-OHA in plasma at concentrations down to 82 pmol/l, with adequate accuracy, precision and scope for further sensitivity. Although this assay would measure 4-hydroxytestosterone (4-OHT) in addition to 4-OHA, the present data suggest that the magnitude of any interference from endogenous steroids and those derived from 4-OHA could only be minimal. A comparison of solvent-extracted and unextracted samples showed that only unconjugated drug was analysed by this radioimmunoassay. A study of plasma protein binding of 4-OHA showed that at therapeutic concentrations, between 13.5 and 16.5% of plasma 4-OHA was not bound to proteins. This assay system could be a useful adjunct to the future development of 4-OHA as an anti-cancer drug.

INTRODUCTION

The cumulative exposure of breast tissue to bioavailable oestrogens plays a major role in the aetiology of endocrine-dependent human breast cancer [1]. In achieving the regression of hormone-dependent tumours, an important approach has been the use of aromatase inhibitors that lower oestrogen production in gonadal and various peripheral tissues [2].

One such inhibitor with promising clinical effects, is 4-hydroxyandrostenedione (4-OHA), which combines competitive inhibition of aromatase activity with inactivation of the enzyme system [3]. This compound was shown to reduce the growth of oestrogen-dependent tumours in rats [4] and was also found to have significant anti-tumour activity in post-menopausal women with breast cancer with few side effects [5].

The future development of 4-OHA as an anti-breast cancer agent will entail extensive studies of optimisation of dose, route of administration and therapeutic regimen. An important part of such investigations would be the monitoring of the drug in body fluids and for this a sensitive and rapid method for 4-OHA analysis has not been reported.

A previously published procedure for 4-OHA comprised chromatographic isolation of the steroid from plasma, followed by radioimmunoassay (RIA) utilizing androstenedione (AD) antibodies with a 25% cross-reactivity with 4-OHA [6]. We previously raised antibodies against 4-OHA in the sheep, the preliminary report of which has been published [7]. Using one of these antisera, a sensitive direct RIA for plasma 4-OHA has now been developed and characterised. These findings are presented here.

*Author to whom all correspondence should be addressed.

EXPERIMENTAL

Chemicals

4-OHA was provided by Ciba–Geigy pharmaceuticals, Horsham, Sussex; 4-hydroxytestosterone and 3β-hydroxy-5α-androstan-4,17-dione was kindly provided by Dr M. Jarman of the Institute of Cancer Research, Sutton. All other steroids were purchased from Sigma Chemicals Ltd.

4-Hydroxytestosterone-17-hemisuccinate (4-OHTHS) was prepared from testosterone-17-hemisuccinate (THS) using a modification of the procedure of Tomoeda *et al.*[8]. Androstenedione (AD) was converted first to 4-hydroxyandrost-4,6-diene-3,17-dione (4-OHΔ^{4,6}A) as described previously by Marsh *et al.*[9]. The product was purified on a silica gel-60 column (70–230 Mesh ASTM, Merck) with benzene:ethyl acetate (1:1 v/v) as the eluant. The 4,6-diene (4-OHΔ^{4,6}A) was then reacted with mercaptopropionic acid, in the presence of sodium methoxide, to produce 4-OHA-7α-carboxyethylthioether (4-OHA-7α-CET). Both 4-OHTHS and 4-OHA-7α-CET were conjugated to ovalbumin using the mixed anhydride method [10].

6,7-[^{3}H]4-OHA was prepared from 4-OHΔ^{4,6}A in the Department of Chemistry, University of Surrey by the method of Marsh *et al.*[9]. The radioactive material was run on a Lipidex-5000 column (Packard Instruments, Caversham, England), 0.7 × 18 cm, with

trimethylpentane:isopropanol (1:5, v/v) as the solvent. [^{3}H]4-OHA was eluted in fractions between 3.25–3.75 ml and the specific activity of the pure material was 15.6 Ci/mmol. It was stored at −20°C.

Mass spectrometry

Mass spectra were kindly recorded by Dr M. C. Dumasia at the Horseracing Forensic Laboratories Ltd, Newmarket. The mass spectrum used for the characterisation of products synthesised in the laboratory was a Finnigan 400 series GC–MS linked to a 6110 series data system, probe: direct insertion heated up to 300°C.

Production of antisera

The conjugate (5 mg) was dissolved in sterile physiological saline (1 ml) and emulsified with 2 ml of non-ulcerative Freund's complete adjuvant (Guildhay Antisera Ltd, Guildford). The emulsion, 3 ml, was injected intramuscularly into the sheep at 6 sites. Blood sampling, by jugular venepuncture, started 9 days after the primary immunisation. Booster injections, (2.5 mg) emulsified with incomplete adjuvant were given at appropriate intervals.

Immunoassay protocol

Approximately 2000 cpm of [^{3}H]4-OHA (in 0.1 ml assay buffer) was incubated, for 1 h at room temperature with 0.1 ml appropriately diluted antiserum, 0.1 ml standard/sample and 0.1 ml 0.05 M phosphate buffer, pH 7.2. Separation of free 4-OHA from the antibody-bound fraction was achieved by the addition of 0.1 ml ice-cold dextran-coated charcoal suspension (2.5%). The plastic LP3 assay tubes were allowed to stand for 10–15 min in iced-water, followed by centrifugation for 10 min at 4°C. The radioactivity in the supernatant (0.4 ml) was measured by scintillation counting.

Plasma samples

The plasma samples used were those from healthy volunteers and from subjects who were given 4-OHA by mouth or intra-muscularly in a study carried out at the Chelsea Hospital for Women, London. These samples from Chelsea will be, hereafter, referred to as "test samples". In the course of assay validation, measurement of 4-OHA was carried out on neat plasma, extracts of plasma and chromatographic fractions of plasma extract. An aliquot of plasma (0.2 ml) was extracted twice with 1 ml diethylether:dichloromethane (4:1, v/v), the extracts pooled and evaporated to dryness under N_2. The residue was reconstituted in 0.5 ml assay buffer and used for analysis. Further resolution of the plasma extracts, when desired, was obtained by chromatography on a Lipidex column, 0.75 × 18 cm, with trimethylpentane:dichloromethane (1:5, v/v) as the developing solvent. The eluate fractions, each 0.35 ml, were monitored for either radioactivity or immunoreactivity with 4-OHA antibodies.

Protein binding studies

The binding of 4-OHA to plasma proteins was determined by the equilibrium dialysis method using small dialysis cells [11]. The plasma sample (1 ml) was placed in one compartment and dialysed against 1 ml of 0.05 M phosphate buffer, pH 7.2 for 18 h at 4°C or room temperature. Immunoreactive or radioactive 4-OHA was measured in the original plasma sample and in each compartment of the dialysis cell.

RESULTS

The 4-hydroxylation of testosterone-17-hemisuccinate was confirmed by the following: (i) the product gave a green colour with ferric chloride, a reaction indicative of the presence of 3-keto-4-ene-4-hydroxy moiety in a steroid molecule [12], (ii) characteristic absorption at 278 nm of 4-OH-3-keto steroids, and (iii) mass spectrum (Fig. 1) with molecular ion at *m/z* 404 and other fragments at *m/z* 304 (M^+—succinic anhydride), *m/z* 286 (304—H_2O), *m/z* 268 (304—$2H_2O$), *m/z* 101 (M^+—4-OHT) and a prominant fragment ion at *m/z* 147 which might incorporate the 4-hydroxylated A-ring [13]. The various chemical features of 4-OH$\Delta^{4,6}$A were in agreement with those previously reported by Marsh *et al.*[9]. Mass spectra of 4-OH$\Delta^{4,6}$A and 4-OHA-7α-CET showed abundant peaks corresponding to their M^+ ions at *m/z* 300 and 406 respectively.

Out of 5 sheep immunised with 4-OHA conjugates, 4 gave a good response. The rise in the antibody titre was highest when, following the priming dose, a period of 35 weeks was given for the serum antibody level to come down to a low value before administering the booster dose of the antigen. After an interval of another 21 weeks a second booster dose was given. Antisera HP/S/1513 and HP/S/A2 raised against the ovalbumin conjugates of 4-OHTHS and 4-OHA-7α-CET respectively, were chosen for the development of the 4-OHA assay.

The assay system was optimized and the protocol is given in Table 1. The affinity constants for HP/S/1513 and HP/S/A2 were 1×10^{11} and 1×10^{10} l/mol respectively. In view of the cross-reactivities of the antisera HP/S/1513 and HP/S/A2 with various steroids (Table 2), the former was chosen for the 4-OHA assay. The antiserum HP/S/A2, reacted markedly with a natural androgen precursor androstenedione (130%) and to some extent (31%) with 3β-hydroxy-5α-androstan-4,17-dione, a major metabolite of 4-OHA in rat hepatocytes [9, 13]. HP/S/1513 on the other hand, showed significant cross-reactivity (60%), but only with 4-hydroxytestosterone (4-OHT), a putative metabolite of 4-OHA. The inter-assay mean dose–response curve from seven successive occasions is given in Fig. 2. The concentration range of the standard curve was between 0.05 and 13 nmol/l. The percentage binding of antigen to antibody at zero

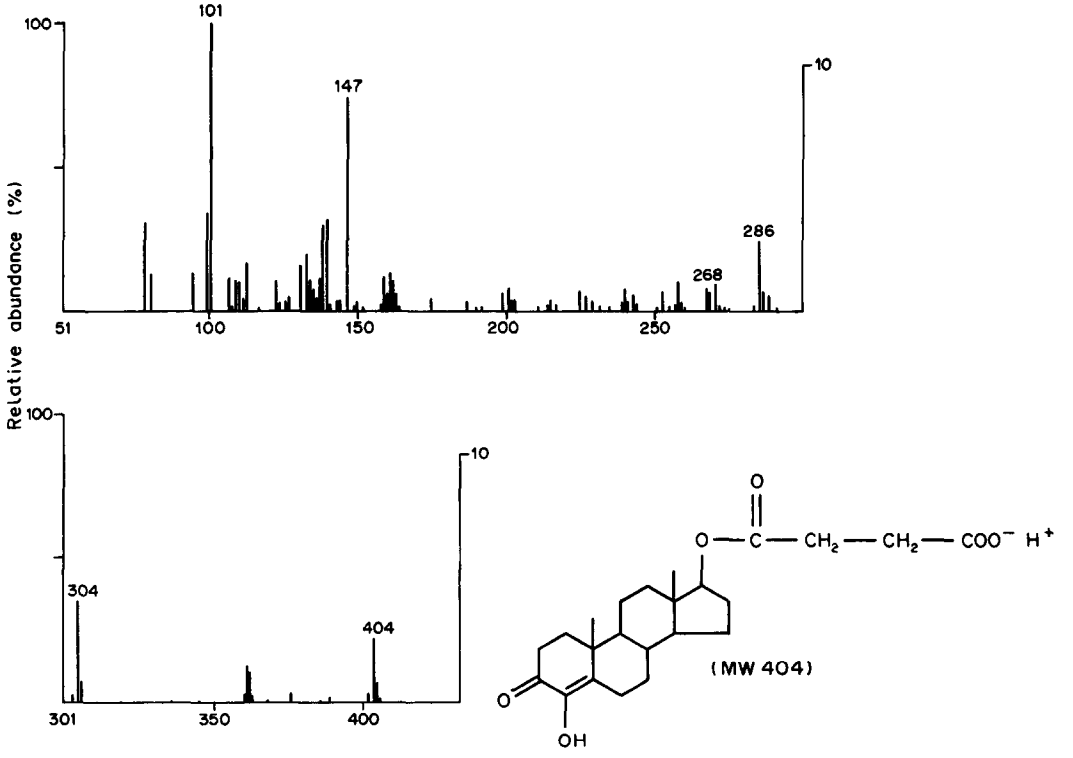

Fig. 1. Mass spectrum and structure of 4-OHT-17β-hemisuccinate.

Table 1. Protocol for 4-OHA radioimmunoassay

Reagent	Volume of reagents added (μl) Total	NSB	Standard/sample tube
Assay buffer	400	300	200
Standard or sample	—	—	100
Antiserum dilution	—	—	100
Label	100	100	100
Incubate overnight at 4°C			
Dextran-coated charcoal (DDC, 2.5%)	—	100	100
Mix and incubate with DCC for 10 min at 4°C, centrifuge at 2500 rpm for 10 min at 4°C.			
Transfer 0.4 ml of supernatant into scintillation vials. Add 4 ml of scintillation fluid (Optiphase safe, Pharmacia-LKB)			

concentration varied between 40 and 60 and the non-specific binding was less than 4%. The assay sensitivity was 82 pmol/l (25 pg/ml) based on 2 SD of the mean of 15 replicate tests of the zero standard and the limit of detection, based on the addition of 10 μl plasma, was 0.8 nmol/l (250 pg/ml). A study of the precision profile over the concentration range between 0.1 and 1.32 nmol/l gave coefficients of variation (CV) under 5%. Up to 20 μl of neat plasma could be used directly without significant matrix effects and no immunoreactivity was detected in normal drug-free plasma. The results of the analysis of 4-OHA-enriched healthy female plasma, at three concentrations and at several dilutions, gave 4-OHA recoveries of 94–96% and CVs of 4.2–7.8% (Table 3).

Table 2. Cross-reactivity of steroids with two 4-OHA antisera

Steroid	% Cross-reaction 1513	A2
4-Hydroxyandrostenedione	100	100
Androstenedione	0.9	130
Testosterone	2.4	<3
4-Hydroxytestosterone	60	<0.8
3-OH-5α-androstan-4,17-dione	0.6	31
4-Hydroxyoestradiol	0.5	—
4-Hydroxyoestrone	0.3	—
Corticosterone	<0.1	—
Cortisone	<0.1	—
Cortisol	<0.1	<3
Oestradiol	<3	<3
Progesterone	0.6	5.8
17α-Hydroxyprogesterone	0.5	—
Deoxy-corticosterone	0.5	—
Dehydroepiandrosterone	<0.3	—

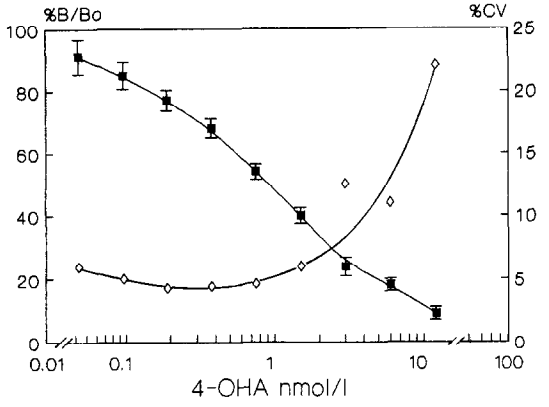

Fig. 2. The mean of seven separate 4-OHA standard curves. (■) concentration points (mean ± SD) of the standard curve (◇) precision profile.

The inter-assay CV at a mean concentration of 0.12 μmol/l, determined in 9 runs over a period of 2 months, was 6%.

Parallelism studies conducted with a plasma sample from a subject who received a dose of 4-OHA with a drug level of 0.11 μmol/l and samples to which the 4-OHA had been added to give levels of 1.65 and 6.62 μmol/l gave a satisfactory correlation between the expected values and those obtained by the present assay (Fig. 3).

Table 3. Intra-assay variation of 4-OHA measurement

4-OHA added	*n*	Recovery (%)	C.V. (%)
3.31 pmol/ml	10	96	4.2
1.65 nmol/ml	10	94	4.9
6.62 nmol/ml	10	95	7.8

In order to confirm that the values obtained with test plasma samples were due to 4-OHA only, direct RIA of a plasma sample was carried out in parallel with assays of the corresponding organic extracts and the collected chromatographic fractions of the extract. On the basis of the recovery of added [^{3}H]4-OHA (>90%), diethylether:dichloromethane (4:1, v/v) was chosen as the solvent for extracting plasma. Plasma sample with added 4-OHA and test samples were analysed by direct RIA and after extraction. On average, the extract gave over 80% of the value from the direct assay (Table 4). When the test plasma extract was separated on a Lipidex column, the elution pattern of immunoreactivity superimposed on that obtained with the corresponding fractions from plasma to which authentic 4-OHA was added and to the elution of radioactivity from [^{3}H]4-OHA fortified plasma (Fig. 4). In each case, 4-OHA was eluted in fractions 9–12; it separated

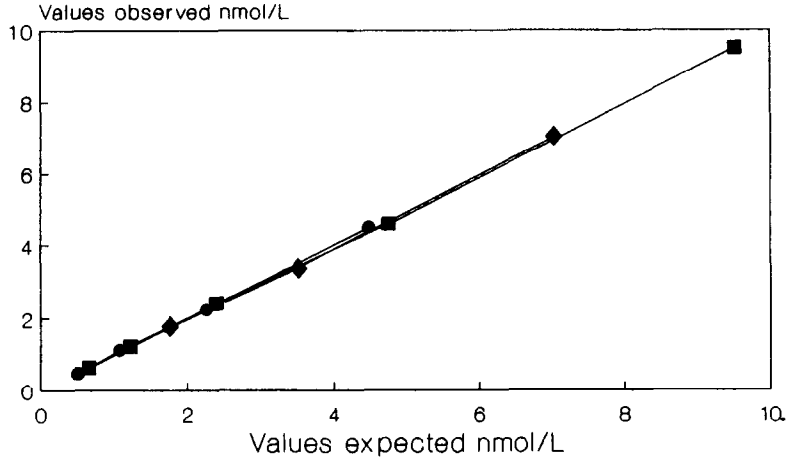

Fig. 3. Parallelism of 4-OHA measurement from plot of expected versus observed values. (●) Patients' plasma (■), spiked plasma 1 (◆), spiked plasma 2.

Table 4. Comparison of 4-OHA values of plasma assayed before and after sample processing

Specimens	4-OHA (nmol/l) Plasma	Plasma extract	Recovery (%)	Column fractions (nmol/l)	Overall recovery (%)
Spiked plasma (No. 1)	99.3	76.1	76	ND	ND
Test plasma 1	49.6	33.1	66	ND	ND
Test plasma 2	132	119	90	ND	ND
Spiked plasma (No. 2)	77.3	63.7	82	59	76
Test plasma 3	16.1	13.7	85	10	62
Test plasma 4	172.6	165	95	114	66

ND = not determined.

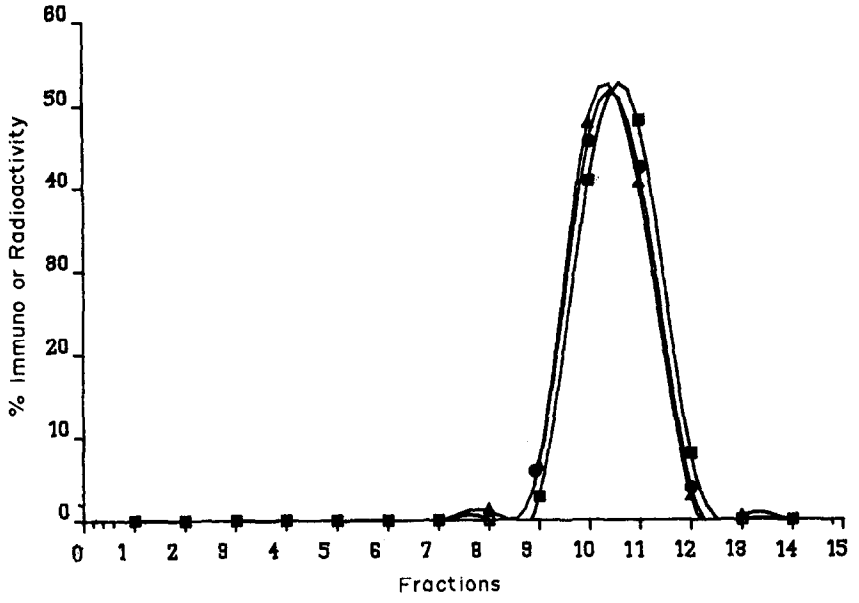

Fig. 4. Column elution profile of 4-OHA. (▲) Normal plasma + [^{3}H]4-OHA (■) normal plasma + 4-OHA, (●) test plasma.

from testosterone and AD (fractions 6 and 7 and 7 and 8 respectively) but there was some overlap with 4-OHT (fractions 8–10). At concentrations between 16.1 and 172.6 nmol/l, in spiked and test samples, RIA of extracts for 4-OHA gave 67–95% of the concentrations found by direct assay of the original plasma (Table 4). The respective values for fractions (9–12) derived from column chromatography of plasma extract ranged between 62 and 76%; in this respect, no marked difference was evident between the test samples and those spiked with 4-OHA. As expected, when RIA with HP/S/A2 was applied to the column fractions from 4-OHT-enriched plasma no significant immunoreactivity was observed. Also, using HP/S/1513 and HP/S/A2, assays on fractions from test plasmas were in good agreement, indicating little or no interference from 4-OHT.

Plasma protein binding of 4-OHA observed in the test samples, as in plasma to which [^{3}H]4-OHA was added, was increased from 85.2 to over 92.5% when the dialysis was carried out at 4°C instead of 20°C. 4-OHA, not bound to plasma proteins, was determined at 20°C in a test sample at 43 nmol/l total drug concentration and in specimens enriched with 4-OHA to 0.15 μmol/l and 1.5 μmol/l levels. The values obtained ranged between 13.5 and 16.5% of the total 4-OHA concentrations in the plasma.

DISCUSSION

Both hapten-ovalbumin conjugates synthesized for this study produced the desired immune response in the sheep. As it has been observed before [14], a prolonged interval between the primary and booster doses of the immunogen, allowing serum antibody concentrations to fall sufficiently to a low steady level, was a common feature to all successful immunisation protocols.

The RIA described here allows the measurement of 4-OHA in plasma at concentrations down to 0.82 nmol/l, with adequate accuracy and precision. Within the assay system, there is still further scope for increasing the sensitivity, but considering the limited available information on plasma 4-OHA levels in patients, that did not seem necessary. After a 250 mg dose of 4-OHA given by mouth, the highest drug concentrations were found after 1–3 h with a mean $\pm$ SE value of 147.2 $\pm$ 24.1 nmol/l [6]. After 24 h the drug was either undetectable ($<$ 0.99 nmol/l) or present in plasma at levels up to 2.6 nmol/l, whereas, two weeks following a 500 mg intramuscular injection, 4-OHA persisted at 1.7 nmol/l level. Unlike the other published method for the measurement of 4-OHA [6], the present RIA can be applied directly to up to 20 μl of plasma without any matrix effects.

The conjugated metabolites of 4-OHA would be water-soluble and are unlikely to be extracted into a mixture of diethylether and dichloromethane. It may, therefore, be concluded from a comparison of results obtained with neat plasma and organic extracts of plasma that mostly unconjugated steroids are measured by this RIA. It is important, because 4-OHA glucuronide has been reported to be the principle urinary metabolite (20 and 45% of dose) in the human and biliary metabolite (42% of dose) in the rat [15]. A plethora of oxidation and reduction products are formed from 4-OHA by isolated rat hepatocytes and these, together with the parent compound as the major component, are rapidly converted

to glucuronide conjugates [13]. Whether the 60% cross-reactivity of 4-OHT with the antiserum HP/S/1513 would limit the use of this RIA for 4-OHA must await a clearer understanding of 4-OHA metabolism in the human. 4-OHT has been found as a plasma metabolite, albeit a minor one in the rat and monkey [9, 16]. However, recently, no 4-OHT was detected in rat hepatocytes as a metabolite either as a conjugate or free steroid [13]. All column chromatographic separations of the extracts of test samples and plasma enriched with appropriate steroids prior to monitoring the collected fractions for radioactivity or immunoreactivity, as may be relevant, showed that the magnitude of any interference with the assay could only be minimal in the samples studied. This does not rule out polymorphic production of 4-OHT in some patients and this RIA combined with a suitable chromatographic procedure may prove useful for assessing the extent and time course of 4-OHT production in man.

An important determinant of drug disposition and clinical efficacy would be the extent to which the drug binds to plasma proteins. Like various endogenous steroids [17], only a small proportion, up to 16%, of 4-OHA present in plasma from subjects who received the drug was found in the free form (not bound to plasma proteins).

Recently, a specific and reliable gas chromatography and isotope dilution mass spectrometry (GC–MS) procedure for determining plasma 4-OHA has been published [18]. It requires 1 ml samples and involves column-extraction, derivative preparation prior to chromatography. The RIA for 4-OHA reported here, following validation against GC–MS, would represent an important step towards the development of 4-OHA for therapeutic purposes. Being a direct, sensitive and robust assay, it has the potential to become an integral part of future clinical trials of 4-OHA. While in the present format, it appears that only the unconjugated drug is measured, the procedure can easily be extended to measure conjugated 4-OHA also by incorporating a step for the hydrolysis of such metabolites.

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