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Oleoyl-estrone is a precursor of an estrone-derived ponderostat signal

Ruth Vilà [a,b], Cristina Cabot [a,b], Laura Villarreal [a,b,1], Ana Monegal [a,b,2], Eva Ayet [a,b,3],
María del Mar Romero [a,b], Maria del Mar Grasa [a,b], Montserrat Esteve [a,b],
José Antonio Fernández-López [a,b], Xavier Remesar [a,b], Marià Alemany [a,b,*]

[a] Department of Nutrition and Food Science, Faculty of Biology, University of Barcelona, Barcelona, Spain
[b] CIBER Obesity and Nutrition, Institute of Health Carlos III, Spain

ABSTRACT

Oleoyl-estrone (OE) is a powerful anti-obesity compound that decreases food intake, decreases insulin resistance and circulating cholesterol. OE stimulates a severe loss of body fat by decreasing adipose tissue lipid synthesis and maintaining lipolysis. Therefore, the body economy loses lipid energy because energy expenditure is maintained. This study analyses the discrepancy between OE effects and the distribution of labelled OE in plasma.

Estrone radioimmunoassay of organic solvent plasma extracts of rats treated with OE showed the massive presence of acyl-estrone, but saponification did not release estrone, but containing similar unknown compound. Analysis of label distribution in plasma after oral gavages of ^{3}H-OE showed the presence of a more hydrophilic compound than OE or any estrogen as well as ^{3}H$_2$O, formed from ^{3}H-OE in the acidic stomach medium. OE was not attached to a specific transporter in plasma. Through serum HPLC analysis we found W, a labelled derivative more hydrophilic than OE or estrone. The results were confirmed using ^{14}C-OE. HPLC–MS/MS studies showed that plasma OE levels were one order of magnitude lower than those of W.

When liver cell cytosols from rats laden with ^{3}H-OE were incubated with nuclei from untreated rats, the OE-derived label (i.e., Ws) was found attached to nuclear DNA. Neither estradiol nor estrone interfered with its binding. W is a fairly hydrophilic compound of low molecular weight containing the estrone nucleus, but it is not an ester because saponification or esterases do not yield estrone as OE does. It is concluded that OE acts through its conversion to W, its active form; which binds to a nuclear receptor different from that of estrogen. The estimated W serum levels are proportional to the pharmacological OE effects *in vivo*. We postulate W as a new type of hormone that exerts the full range of *in vivo* effects thus far attributed to OE. The full identification of W is anticipated to open the way for the development of new OE-like anti-obesity drugs.

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1. Introduction

1.1. Oleoyl-estrone, nature and effects

Oleoyl-estrone (OE) is a powerful slimming agent [1,2]; it decreases food intake but, at the same time, maintains energy expenditure [3]. The energy gap is closed by the mobilisation of white adipose tissue (WAT) reserves [4], sparing body protein [3]. OE also decreases the high insulin [5] and hyperlipidemia of obese rats [6], particularly of cholesterol [6,7]. Liver glycogen is maintained (or increased) by OE even under a severe loss of body lipid energy [5], also maintaining normoglycemia [8].

Treatment with OE is more effective in male than in female rats [9] and induces weight losses even under treatment with cafeteria diet [10] or other hyperlipidic diets [4,11]. A peculiarity of OE treatment is the permanent loss of fat, suggesting a role as ponderostat

signal for the estrone ester [12]. When OE is given in combination with a β_3-adrenergic agonist, the WAT wasting effects are markedly increased [13], with additive effects [14]. Similar, but less marked, additive effects were also observed with dexfenfluramine, sibutramine [15] and a thiazolidinedione [16], with opposing effects on WAT lipid integrity, but coincident in maintaining glycemia. In fact, OE slightly decreased UCP1 expression in brown adipose tissue [17], but also increased the expression of this uncoupling protein in some WAT sites [14].

The effects of OE on lipids can be summarised in the inhibition of lipogenesis in WAT [18] (but not in liver, which has sufficient glucose available) [19]. These effects include decreased lipoprotein lipase expression and activity in WAT (and hepatic lipase in liver), but increased activity in muscle [6], whilst intracellular lipolysis is unaffected [18]. SREBP1c is involved in mediating these effects [19]. In the case of the liver, lipogenesis is exacerbated by corticosteroids, which revert the overall wasting of energy induced by OE [20]. Oleoyl-estrone induces a counter-regulatory increase in ACTH and corticosterone levels [21], with increased corticosteroid activity and turnover [22]. This increase is largely due to adrenal hypertrophia and increased expression of the entire corticosteroid synthetic pathway [23] but is also due to increased liver corticosteroid disposal via 5α-reductase [22]. Treatment with OE reduces the WAT expression of most cytokines [5,14], especially leptin [5]. The expression of adiponectin is also decreased by OE [14]. Although the effects of OE on WAT are fairly diverse depending on the WAT site, one effect of OE is apoptosis [24]. Table 1 shows the principal effects of OE treatment and its metabolic actions.

1.2. OE methodology

OE is a wax-like lipophilic ester that tends to attach to any surface (including dissolved proteins) in aqueous media. OE is particularly non-reactive and is a difficult target for ionisation and analysis by mass spectrometry. In addition, characterisation of its estrogenic moiety is not an exclusive OE marker because plasma and tissues contain a number of estrogens with similar derivative-forming reactions. The oleoyl moiety is even less promising because of its high abundance in rat lipids [24].

We developed a method that allowed us to measure estrone (fatty) acyl-esters: after organic extraction of plasma, the dried lipid extract was saponified with hydroalcoholic KOH, and the released free estrone was again extracted and analysed by radioimmuno-analysis (RIA) [25]. A modified method was used for the analysis of acyl-estrone in foods [26]. A much improved version of this method was developed for the estimation of acyl-estrone in dairy products [27]. An antibody that does not react with estradiol was used to measure the evolved estrone and allowed us to assign the levels of "acyl-estrone" measured to OE. In fact, values measured in rats injected with OE were higher than those of controls [5,12,28].

Previous MS studies have shown that OE is present both in plasma and adipose tissue fat [3], but its low ionisation makes its detection and analysis a difficult task.

1.3. Specificity of OE and objectives of the present study

The idea of specificity of the molecule to elicit the observed marked metabolic changes was further analysed by preparing a number of structurally similar molecules (mainly estradiol or estrone fatty acid esters) [29]. The results showed that the sought in vivo effects were optimal only for OE. Saturation of the fatty acid, or its shortening, hydroxylation in C17 or change of union (ether, amide) of the acyl moiety with estrone all resulted in decreases or loss of slimming effectiveness. Only oleoyl-diethyl-stilbestrol gave similar results to OE in vivo [29].

Table 1

Estimation of [14]C- and ^{3}H-labelled OE derivative (containing the estrone nucleus) in plasma of adult male rats subjected to a gavage of 10 nmol/g OE 2 h before the analysis.

Parameter	Units	^{3}H-OE ($N = 3$)	^{14}C-OE ($N = 4$)
OE dose given orally (3000 nmol)	MBq	3.96 ± 0.41	0.952 ± 0.101
Specific activity of the gavage	Bq/nmol	1319 ± 138	317 ± 34
Plasma label (discounting blanks)	Bq/mL	2190 ± 1070	130 ± 40
Estimated "estrone" label concentration in plasma	nM	1626 ± 641	417 ± 144
Rat body weight	g	351 ± 5	295 ± 36
Label remaining in the stomach and intestinal contents	MBq	1.23 ± 0.13	0.295 ± 0.031
Label absorbed	Mbq	2.73 ± 0.28	0.657 ± 0.070
Body label in the form of tritiated water	%	11.8 ± 4.3	–
Specific activity of circulating "estrone"	Bq/nmol	1026 ± 124	317 ± 34
"Estrone" label concentration in plasma discounting tritiated water and correcting for final specific activity	nM	404 ± 204	417 ± 144
Mean "estrone" label concentration in plasma	nM	413 ± 144	

In spite of all indirect proof and the specificity of the link between OE structure and physiological/pharmacological effects, the application of the described methodology produced a number of inconsistencies when the measured levels were cross-analysed with all of the available data:

(a) The majority of the problems posed by the extreme lipophilia of OE are likely overcome in biological systems by the binding of OE to a protein transporting system, analogous to those of a number of steroid hormones [30]

(b) Early analyses of plasma using mass spectrometry showed the presence of OE, but at much lower levels compared with the RIA-derived data.

(c) The consequences of a change in the supplier of the estrone antibody (the one we used was discontinued) were impossible to overcome, as all new antibodies were ineffective. The analytical results were no longer reproducible.

(d) The use of tritium-labelled OE (in the estrone moiety) indicated that in plasma, the levels of label (whose only source was the OE estrone moiety) were fairly higher than the measured levels of "acyl-estrone", even when other estrone-containing molecules (free estrone, estrone sulphate) were taken into account.

The main objective of this study was to clarify a number of methodological inconsistencies that seriously hindered the analysis of the mechanism of action of OE. Initially, the search was centred on the OE receptor and on the carrier "transporting protein" that allowed such a lipophilic molecule as OE to be transported in the hydrophilic plasma medium, even considering its attachment to lipoproteins [31]. However, the key aim was to understand whether OE acts as a signal by itself or is transformed into a more specific – and powerful – signal before being transported by the bloodstream and acting on OE target tissues.

2. Experimental

2.1. The search for a plasma OE-transporter

2.1.1. In vivo analysis of labelled OE in plasma of rats receiving labelled OE gavages

Large male Wistar rats (400–450 g) were used. All animal handling procedures were approved by the Animal Management Ethics Committee of the University of Barcelona and were in full compliance with the norms and directives of the EU, Spain and Catalonia. The rats were given the standard working OE dose: a single 10 nmol/g gavage of OE (OED, Barcelona, Spain) in 0.2 mL of sunflower oil [32]. The rats were then killed by decapitation, and the blood was recovered and allowed to clot. The serum was then separated by centrifugation, frozen and stored at −80 °C until needed. Other rats (controls) received the vehicle alone. Tritium-labelled OE was synthesised by us [3] from ^{3}H-estrone (NEN, Bad Neuheim, Germany) and oleoyl-chloride (Sigma).

The i.v. [33] or oral [34] administration of labelled OE resulted in an increase of circulating label in plasma. The esterified estrone content of plasma in OE-laden rats also dramatically increased [35]. Similarly, we found that the proportion of circulating acyl-estrone was increased in humans with higher body fat content [36], but not in the morbidly obese [37]. The extreme lipophilic nature of OE precludes its simple transport as such, which is a problem shared by triacylglycerols, cholesterol and even non-esterified fatty acids. Consequently, we searched for an OE-carrier protein or OE transporter (OET).

2.1.2. OE and lipoproteins

The close relationship of OE with lipoprotein fractions [31,38] and its relative binding to these lipoproteins were countered by the easy washing out of label from native plasma lipoprotein fractions from rats loaded with ^{3}H-OE, separated by size exclusion chromatography in Sephacryl S200 columns using a 50 mM phosphate buffer pH 7.2 containing NaCl 150 mM. Most of the label was eluted with the front, and the label was retained in the high MW protein fractions (i.e., lipoproteins). When repeatedly passed through the column, the label increasingly eluted with the front and finally could be entirely recovered in the low MW section in spite of its initial attachment to lipoproteins.

Classical lipoprotein separation is performed through ultracentrifugation in a saline gradient [39]. However, this approach results in the distribution of OE label between the main lipoprotein classes and the lipoprotein-free serum fraction [38,40]. These data suggested that the OET was indeed a small MW protein naturally attached in a loose way to larger lipoproteins but was easily washed out of them. This behaviour hinted at both small size and a relative polarisation with lipophilic and hydrophilic domains.

OE can be easily separated (and visualised and counted) from estrone by TLC on silica gel plates using a mobile phase of hexane/ethyl ether/acetic acid (20:5:1) because the rf for estrone in this system was 0.25 and 0.52 for OE. Analysis of plasma organic phase extracts showed practically no estrone nor OE, with most of the label remaining at the origin. This result was initially interpreted as proof that most of the OE in plasma was bound to OET and was unable to elute in such a hydrophobic running phase. We already expected low estrone levels because plasma estrone is in the 0.2–2 nM range [35,41]; the values expected for OE were much higher [35].

2.1.3. In vitro loading of plasma with labelled OE

Celite (World Minerals, Rubí, Spain) was suspended in a methanol suspension of ^{3}H-OE, and after 1 h incubation, the methanol was evaporated by centrifugation under vacuum. The high surface area of celite was thus heavily impregnated with OE; repeated washings in water resulted in negligible losses of label. A suspension of ^{3}H-OE-celite was added to rat plasma and incubated for 12 h at 35 °C. The objective was to allow OE to be loaded to OET (or interchanged with cold OE-OET). Extraction of the plasma with trichloromethane–methanol (2:1) or hexane resulted in the practical washing of all label to the organic phase. Cold-ethanol precipitation [42] also yielded a Cohn's fraction III supernatant practically devoid of label.

However, extraction of plasma from ^{3}H-OE-laden rats with trichloromethane/methanol (2:1) [43] resulted in the retention of only 42% of the label in the organic phase, in contrast with 81% for a suspension of labelled OE (of about 80–85% purity) in water, with the rest remaining in the aqueous phase.

2.1.4. Plasma protein fractioning and OE

Separation of plasma fractions using cold ethanol, according to the classical Cohn procedure [42], showed that most of the plasma label remained in the supernatant of Cohn's fraction III. The OET was soluble in ethanol/water 1:1. Extraction of this supernatant with trichloromethane/methanol diluted with water yielded a 42% label in the organic phase, which was the same as the result that was obtained with native plasma. Extraction of plasma using a more apolar solvent, hexane, resulted in lower label yields (23%) in the organic phase; however, this recovery decreased to only 6% in the Cohn's fraction III supernatant, which are values that are markedly different from those of OE.

Using either the Cohn's fraction III supernatant or the front eluates of Sephacryl S200 columns (Sigma) from ^{3}H-OE-laden rat plasma, a number of protein identification analyses were performed:

(1) Lyophilisation resulted in the loss of variable amounts of label, but in general only about 60% of tritium was lost. It was assumed that this was simply tritiated water, confirmed later as explained further on this paper. The remaining label corresponded roughly to that extracted with organic solvents.

(2) The lyophilised extract was subjected to 2 h of heat (150 °C) in order to denature the OET. However, TLC separation of the organic extract showed that neither estrone nor OE was present, and all label remained unchanged at the origin. It is almost impossible for a protein to maintain its structure unaltered and remain bound to its ligand when subjected to such high temperatures.

(3) The use of other protein-denaturing agents, such as perchloric acid, tannin, trichloroacetic acid, or heavy metal salts (uranylnitrate, lead acetate), yielded the same results: OE was not released. Most proteins are denatured and release their ligands when subjected to at least a few of the denaturing agents listed.

(4) The addition of acetone (2:1) resulted in no precipitate. Acetone extracts of plasma also yielded all label in the supernatant (after correcting for the label occluded with solvent in the precipitate). Proteins and peptides precipitate under high acetone concentrations.

(5) Analysis of these supposed OET fractions by MALDI-TOF showed no results at all; i.e., no protein or compound with a MW higher than 1 kDa was present in the samples analysed.

2.1.5. Protein separation and fate of plasma OE-derived label

Ultrafiltration through molecular sieves of 10 kDa and 3 kDa of both Sephacryl eluates and Cohn's fraction III supernatants resulted in practically all label passing through. Ultrafiltration of plasma diluted with saline resulted in the retention of a small but highly variable proportion of label (13–34%), which was probably loosely attached to large lipoproteins.

Polyacrylamide gel electrophoresis of concentrated Sephacryl eluates and Cohn's fraction III supernatants (as well as of crude plasma) from rats treated with ^{3}H-OE resulted in practically all of the label remaining in the starting wells. The rest of the label (probably tritiated water) was diffusely distributed in the buffer front, in zones with no significant protein presence (as assessed by AgNO$_3$ or Coomassie blue staining).

The data gathered clearly demonstrated that there was no free OE in plasma and that OE was not carried by a specific protein transporter. In addition, a sizeable part of the label, lost by lyophilisation, may correspond to tritiated water or a similar volatile compound of low MW and is probably unrelated to OE function and effects. OE behaves in very different ways with respect to solvent extraction than the compound(s) bearing the tritium label which we can consider directly related to the physiological or pharmacological actions of OE. This active form of OE has a very low MW, is more hydrophilic than OE and is thermically stable, but can nonetheless be extracted with organic solvents.

2.2. HPLC–MS/MS analyses

2.2.1. Differentiation of OE and estrone

To clarify the situation, we decided to measure OE by HPLC–MS/MS, using two different strategies: (a) the saponification–derivatisation system used previously, but instead measuring dansyl-estrone by HPLC–MS/MS and (b) searching for methodological approaches or conditions that allowed us to ionise – albeit partially – the OE molecule and/or break it up in the mass spectrometer and compare the fragments' family with that of OE.

The study was first developed using pure standards. Previous analyses showed that about 98–99% of ^{3}H-OE was extracted with the organic phase, almost irrespective of the solvent used. Standards of OE, estrone or other estrogens (Steraloids, Newport, RI, USA, and Sigma, St Louis, MO, USA) were used; deuterated estradiol and estrone (CDN Isotopes, Pointe Claire, ON, Canada) were also used as internal standards. Chromatographic-grade solvents were from Riedel de Haën (Seelze, Germany). Deuterated OE was synthesised [3] from oleoyl-chloride and deuterated estrone and later purified by normal phase chromatography on silica columns (eluted with hexane ethyl acetate/acetic acid; 19/1/1). The rf for estrone was 0.22, that of OE was 0.55 and oleic acid was 0.73. The structure and integrity of OE was checked by NMR dissolved in CDCl$_3$.

We used a LC–MS/MS (Varian, Palo Alto, CA, USA) with an instrumental setup consisting of: 210 pump with an on-line degassifier, 410 autosampler and 1200L triple quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source operated in positive ion mode.

Full identification of the OE and estrone peaks was performed by changing the conditions of elution and the sensitivity/ionisation and breakup of the molecules. In the final (definitive) analytical method developed, chromatographic separation was carried out using an Atlantis column 50 mm × 2.1 mm × 3 μm (Waters, Milford, USA). The solvents were: (A) 10 mM ammonium formiate in 1 g/L formic acid and (B) 1 g/L formic acid in acetonitrile. The flow rate

was 0.3 mL/min. Standards of OE and estrogens were separated using this system as shown in Fig. 1. However, the concentrations needed to show measurable peaks were much higher than those found in serum for most estrogens. Consequently, a modified dansyl-chloride (Sigma) derivatisation method was used to measure the serum levels of estrone and its oxidised derivatives [44].

2.2.2. Analysis of serum samples

Triplicate serum samples (0.400 mL each) were used; one of the samples was duped with 0.010 mL of 50 nM deuterated OE (d4-estrone), 50 nM free d4-estrone or 25 nM free d4-estradiol as internal standards. These samples were extracted with 2 mL of chilled dichloromethane, and after centrifugation, the organic phase was dried under a gentle stream of nitrogen. The dry residues were dissolved in 0.200 mL methanol, and 0.020 mL was injected into the HPLC.

OE was measured in serum without derivatisation, assuming (from the RIA-based method data) that its concentration in plasma was in the 100 nM range. The final conditions for OE direct analysis in plasma organic extracts allowed the measurement of concentrations >20 nM. Under these conditions, no OE was found in rat serum from either rats having received an OE load or controls. Using dansyl chloride derivatisation, the sensitivity improved considerably; total estrone and estradiol levels were detectable (albeit low because the serum donors were male). Total estrone (i.e., free and sulphate) was in the 1–6 nM range (males) or 5–6 nM (females) in controls, with the somewhat higher values of 5–16 nM (males) and 5–11 nM (females) in OE-treated rats. Saponification with KOH raised the recovery of estrone to 25–32 nM (males) and 33–35 nM (females) in controls and 49–61 nM (males) and 43–70 nM (females) in OE-treated rats.

Serum samples (400 μL) were extracted and dried as indicated above, but dissolved in only 0.050 mL of methanol of which 0.04 mL was injected into the HPLC. These samples allowed us to detect OE in serum at the limit of sensitivity of the system. The concentrations (N = 6) were 10–20 nM for controls and 11–22 nM for OE-treated rats. These differences were not considered significant because both ranges were practically superimposed.

2.2.3. OE derivatisation

OE was also derivatised using the Girard P reaction [45]. This procedure allowed a decrease in the limit of detection of OE (derivatised) in plasma through HPLC–MS/MS to about 50 nM using 100 μL samples of plasma. We used Inertsil ODS3 (Varian) columns 150 mm × 30 mm × 3 μm, eluted with (A) 1 g/L formic acid in water and (B) acetonitrile at a flow of 0.3 mL/min. The method was checked with standards of deuterated OE and calibration curves were developed. Using this approach, the levels of OE measured in a trichloromethane:methanol extract of the plasma of Wistar rats were in the 20–30 nM range, showing considerable variability and results that were not different from those of rats that received a standard oral load of OE. These values were in the same range as data previously published using a similar extraction–derivatisation method (ranging from <5 nM to 54 nM in human samples) [46].

The obvious conclusion was that OE was in limited amounts in normal serum, and the OE gavage did not change its scant presence. However, the OE load also did not result in a sizeable increase of estrone, its esters or hydroxylated derivatives.

2.3. Estrone radioimmunoanalysis

2.3.1. Radioimmunoanalysis-based method for serum acyl estrone esters

The use of a RIA (with an antibody that does not react with estradiol) to measure the evolved estrone convinced us of the prac-
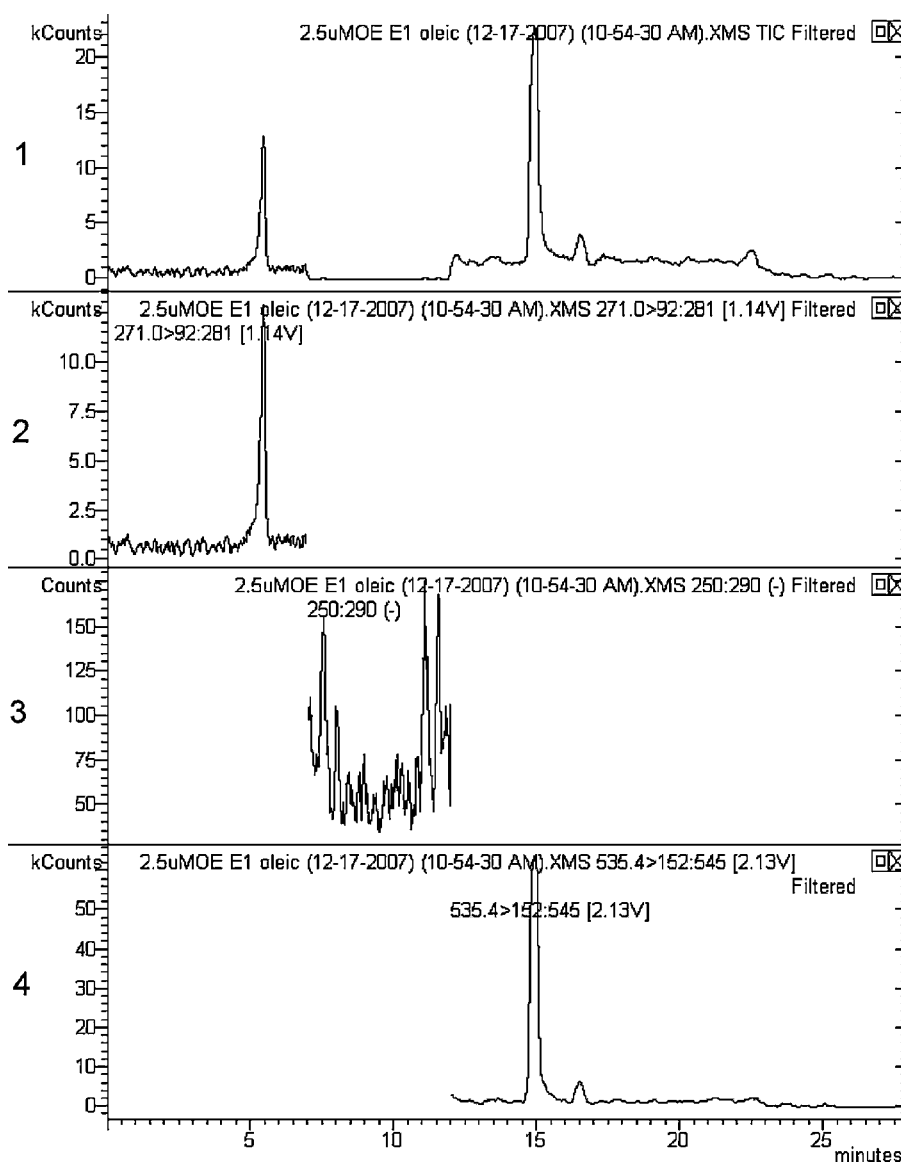
Fig. 1. HPLC–MS/MS chromatogram of OE, oleic acid and estrone standards. All separate standards were added at the same concentration, 2.5 μM. The conditions for separation are given in the text. The main breakup fragments are indicated in the figure.

tical identity of OE and "acyl-estrone", which was reinforced by the increased levels observed in rats injected with OE [5,12,28].

The use of a specific RIA in the final phase of the analytical method eliminated, at least theoretically, most of the possible interferences by other compounds. The only significantly plausible interference in the use of the specific anti-estrone antibody (E3153, Sigma) was cross-reactivity with dehydroepiandrosterone sulphate. This compound was removed from human plasma samples because of its high levels, but not from most rat samples because of its much lower concentrations in rat plasma compared with human plasma [47].

2.3.2. Specificity of antibodies

RIA is a very specific method for analysis of a given molecule in a complex mixture, provided that the antibody is adequate (reactive, specific, sufficiently high titer, low cross-reactivity). When our supply of "good" estrone antibody was exhausted, we obtained and compared all estrone kits and antibodies we were able to find. However, the low supply and variety of sources of antibodies against estrone is a problem resulting from estrone no longer being a med-

ically interesting molecule. Estrone is seldom analysed in humans, and in the recent past, a sizeable number of the few animal studies using this compound were ours; consequently, the commercially available supply of antibody is low. In addition, optimised modern RIA and ELISA methods rely on estrone antibodies raised against derivatives of the estrone nucleus with side chains attached in the C5–C6 region. Neither OE nor the estrone freed by the saponification of serum from rats pre-loaded with an OE gavage reacted with these antibodies. The estrone obtained by the saponification of pure synthetic OE, however, was easily measured using almost all methods we tested.

Because our early studies have shown the presence of "estrone" in saponified plasmas (the methodology also detected free estrone, which we measured using the same setup), but the new (and more sophisticated) antibodies/kits were not reactive (but they reacted with free estrone notwithstanding), we assumed that the earlier (i.e., discontinued) antibody was able to react with an unknown molecule with a marked structural similarity to estrone. This reaction was not reproduced by the newer and more specific antibodies. The high cross-reactivity of the old antibody with DHEA and estrone

sulphate was also not reproduced in the modern kits, which largely discriminate estrone from estrone sulphate and show low cross-reactivity with DHEA–sulphate.

The data allow us to postulate that after the saponification of organic phase-extracted serum lipophilic fractions, there was not the expected release of estrone, or at least an estrone moiety recognisable by the "modern" specific antibodies. However, the relatively high (in the $0.1\,\mu M$ range) earlier measured levels of "estrone" suggest that – even provided that reactivity with real estrone was higher – the levels of this thus far unknown compound freed by saponification should be fairly high, at least in the range of our earlier analyses. This unknown entity, evidently not estrone, must also possess a structure with sufficient similarity to estrone as to confound a relatively specific antibody.

2.4. In vivo tracer studies with labelled estrone

2.4.1. Plasma label after OE gavages, separation with exclusion size columns

The OE gavage approach described above (including size and handling of the animals and samples) was repeated using labelled OE, which we synthesised from labelled estrone and oleoyl chloride [3]. Using tritium-labelled estrone (NEN), we produced ^{3}H-OE to label the gavage doses to a mean specific activity of 1.3 kBq/nmol, i.e., giving 3.9 MBq of labelled OE to each rat. This way, we obtained approximately 2.2 kBq/mL in the serum of rats under the standard gavage conditions described above.

The data calculated from the label content in plasma or serum (using the specific activity of the gavage dose) were in the low micromolar range, i.e., more than 3 orders of magnitude higher than the direct measurements with the HPLC–MS/MS. Evidently, the tritium label was not attached to OE.

Serum components of rats loaded with ^{3}H-labelled OE were separated through columns of Sephacryl S200 (Sigma). A small fraction of the label eluted with high MW proteins (we assumed they were lipoproteins), but most of the label was eluted in a broad peak running with the solvent front. This peak did not correspond to OE or to any other tested estrone derivative. Using Sephadex G100 columns (Sigma), we obtained a small proportion of the label eluting with the front, i.e., with high molecular weight proteins and any lipophilic compound attached to them. However, most of the label eluted with the void-volume of the column.

Using the Varian HPLC with a Zorbax GF-250 molecular-sieve column (4.6 mm × 250 mm, 4 μm; Agilent) and a 50 mM phosphate buffer pH 7.5 containing 150 mM NaCl (0.5 mL/min), we proceeded to elute crude serum samples from rats that had received a tritium-labelled OE gavage. The HPLC eluates were counted in 0.2 mL aliquots in a scintillation counter. Fig. 2 shows the presence of tritium in the eluate fractions. Most of the label was carried practically with the front; smaller peaks were eluted later, including estrone, estradiol and several unknowns. The same pattern was observed in controls and in rats receiving a gavage of only the tracer (no cold OE). Serum samples with no tritium label were also run through the HPLC using the same setup, with the eluate being analysed on line by MS/MS. No significant ion counts were found in the time fraction corresponding to the massive tritium peak.

Crude serum plasma from rats that had received a gavage of ^{14}C-OE 2 h before sacrifice were run on Sephadex G100 (Sigma) columns. The distribution of the label can be observed in Fig. 3. Extraction of the main peak with a SPE C18 cartridge (SEP-PAK, Waters) resulted in the retention of >99% of the label, which was quantitatively eluted with methanol. Repeating the same experiment with tritiated-OE, the main peak (coincident in time with that of ^{14}C-OE and close to the void volume of the column) was extracted with a SPE C18 cartridge; however, only 13% of the

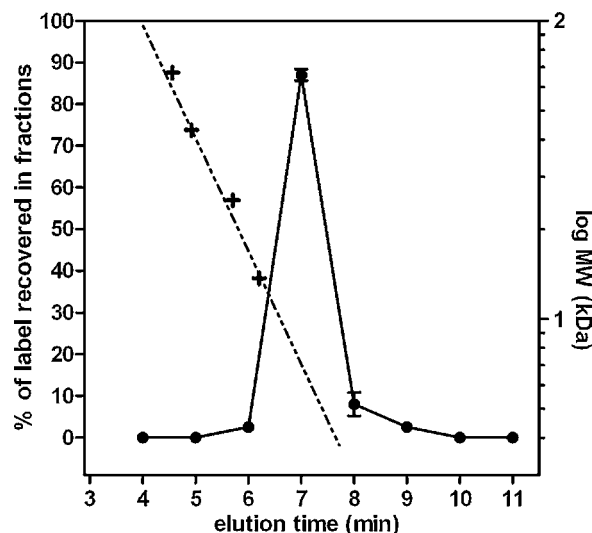**Fig. 2.** Distribution of ^{3}H label in the serum of a male overweight rat 2 h after a ^{3}H-OE gavage. The data correspond to HPLC eluates of sera using a Zorbax GF-250 molecular-sieve column. The crosses and dotted line represent the (log) relationship between molecular weight and elution time obtained from a series of protein standards.

label was retained, the rest being washed away in the aqueous fraction. The retained label was again quantitatively eluted with methanol.

2.4.2. The problem of loss of label from tritium or deuterated estrone

Saponification of pure deuterium-labelled OE resulted in a significant loss of deuterium from the estrone moiety [48]. This was a consequence of the interchange of D and H from estrone to the solvent in the highly alkaline medium. We observed similar results in strongly acidic media used to test the acidic hydrolysis of OE.

Tritium-labelled OE, and free estrone, also lost label to the medium under extreme pH conditions in highly variable proportions. After oral gavage, a high proportion of OE (essentially the part of the gavage dose not hydrolysed by esterases and therefore taken up by the animal) remains in the stomach and its walls [33]. Because stomach pH is in the 0–1 range, we can expect a sizeable interchange of tritium from OE with stomach juices, mainly water. Because body water interchange is faster than the passage of lipophilic compounds (OE, estrone), we can expect that a large
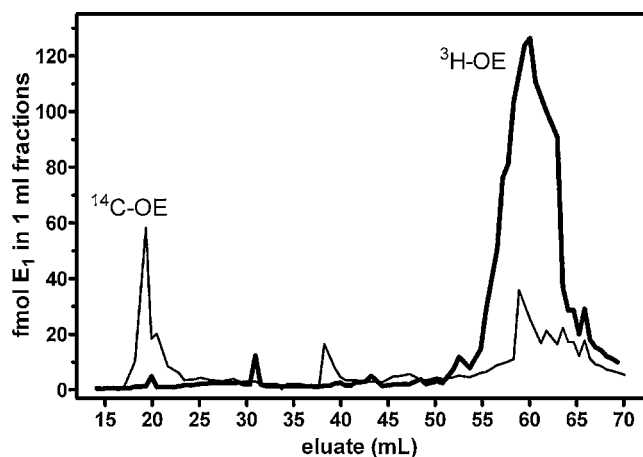

Fig. 3. Distribution of label in plasma of rats after receiving gavages of ^{14}C- or ^{3}H-labelled OE eluted from a Sephadex-G100 column. The data are expressed in fmol/per 1 mL fraction.

Table 2
Mean plasma concentrations of OE and related compounds 2 h after an oral gavage dose of 10 nmol/g OE to adult male Wistar rats.

	nM
Plasma "estrone" label concentration (Table 1)	413 ± 144
Plasma "acyl-estrone" concentration (extraction + KOH + RIA)	758 ± 442
Plasma "acyl-estrone" concentration (as above but discounting estrone sulphate)	576 ± 335
Plasma free estrone (RIA)	0.860 ± 0.026
Plasma estrone sulphate (RIA)	181 ± 38
Plasma free 17β-estradiol (RIA)	2.32 ± 0.09
Mean OE concentration in plasma (Gir-P, MS)	61 ± 15

The data correspond to different experimental setups and different animals.

part of body water would become tainted by the tritiated water in the gut lumen.

Lyophilisation of plasma resulted in the loss of most of its radioactivity. Distillation *in vacuo* using Thunberg tubes yielded tritium-labelled water extracted from plasma. Approximate estimations of the proportion of label present in serum in the form of tritiated water (or other volatile solvents) were in the range of 78–83%. Assuming a water-accessible volume of water in the rat of about 75% (i.e., 280 mL for a large male rat) and a gavage of 4 MBq, the amount of tritiated water formed in 2 h was approximately 477 kBq. This result leads to a mean specific activity of the whole body water of 1.7 kBq/mL, which is approximately $11.8 \pm 4.3\%$ of the total label in the gavage dose (Table 2). These values are maximal and are rough estimations that do not take into account the faster accessibility of the plasma space and the longer time needed to reach all corners of intracellular and interstitial water compartments. In any case, the high proportion of label found in the front-running peak agrees with the notion that most of the serum tritium was in the form of tritiated water.

Consequently, all calculations based on total label content in plasma (or even those of specific peaks in which the initial specific activity was used) could no longer be applied because of the artifactual presence of large amounts of tritiated water (Fig. 4).

2.4.3. Use of ^{14}C-OE in tracer studies

The obvious substitute for tracer studies was ^{14}C-OE. This stable label had the advantage that no interchange of labile deuterium or tritium label was possible even under extreme conditions, unless the molecule itself was destroyed. This approach also allowed for more precise measurements of radioactivity with only minimal errors due to quenching. However, use of ^{14}C-OE posed other significant problems: very scant commercial availability, high cost and low specific activity (which are consequences of the difficulty in
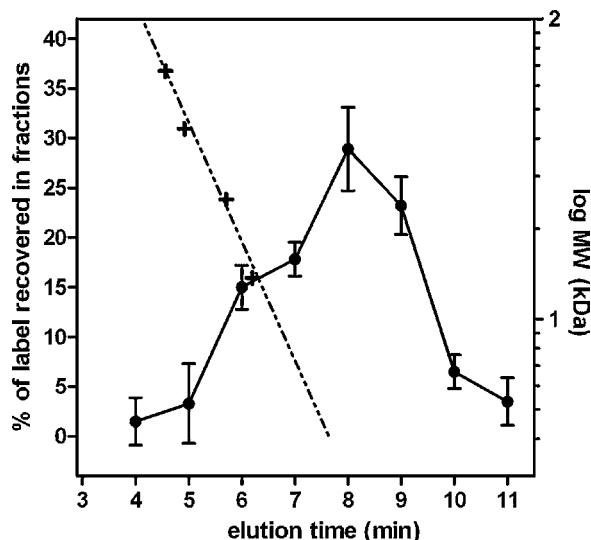

Fig. 5. Distribution of the ^{14}C label in the plasma of male overweight rats 2 h after receiving a gavage of ^{14}C-OE. The data correspond to eluates of sera after HPLC using a Zorbax GF-250 molecular-sieve column. The crosses and dotted line represent the (log) relationship between MW and elution time obtained from a series of protein MW standards.

incorporating ^{14}C, usually in the C4 position) and its longer half-life. However, using the very limited amount of ^{14}C-estrone available (Tjaden Biosciences, Burlington, IA, USA), the oral-gavage procedures were repeated as described for ^{3}H-OE.

The results, from rat sera chromatography using a Zorbax GF-250 molecular-sieve column, obtained with ^{14}C-OE, were markedly different from those of ^{3}H-OE (Fig. 5). Instead of a huge peak in ^{3}H-OE sera at 7 min we obtained a more flattened ^{14}C-OE sera image, with a main peak at 8 min and a secondary peak at 6 min. Application of a linear protein MW series of standards would give a MW of about 5 kDa for the ^{3}H-OE rat peak and a similar value for ^{14}C-OE, plus larger peaks out of range. This is probably a consequence of the lipophilic retention activity of the Zorbax GF-250 columns in addition to its simple MW separation. The largest tritium peak was coincident with the void volume of the column and fully corresponds to tritiated water, obscuring the other peaks because of its size.

Running cold sera of rats receiving the same gavage and treatment (but no label), the MS/MS analysis of the eluates was possible. Two early small peaks and a broader undefined zone of label distribution eluting late were observed (Figs. 6 and 7). The last zone could be roughly approximated to estrone, but there was no clear coinci-
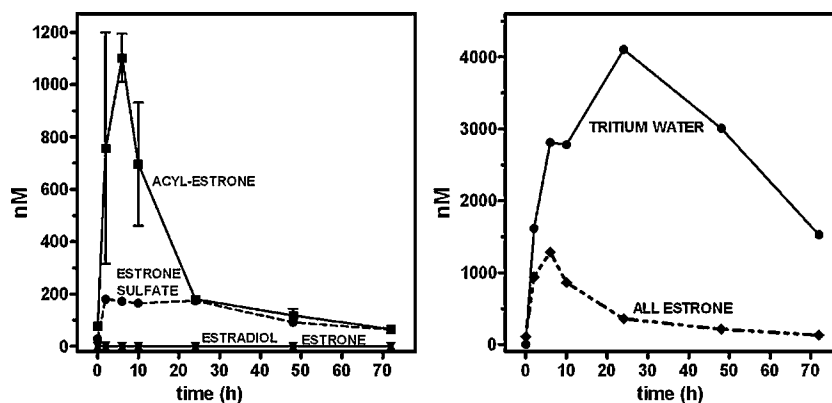
Fig. 4. Distribution of plasma estrone and related molecules in male rats after a single oral gavage of OE. The graph on the left presents the values obtained for "acyl-estrone" (using the extraction, saponification and RIA method), estrone sulphate (RIA), estradiol (RIA) and estrone (RIA) in nM. The graph on the right presents the combined estrone compounds compared with the calculated label corresponding to tritium water, also in nM.
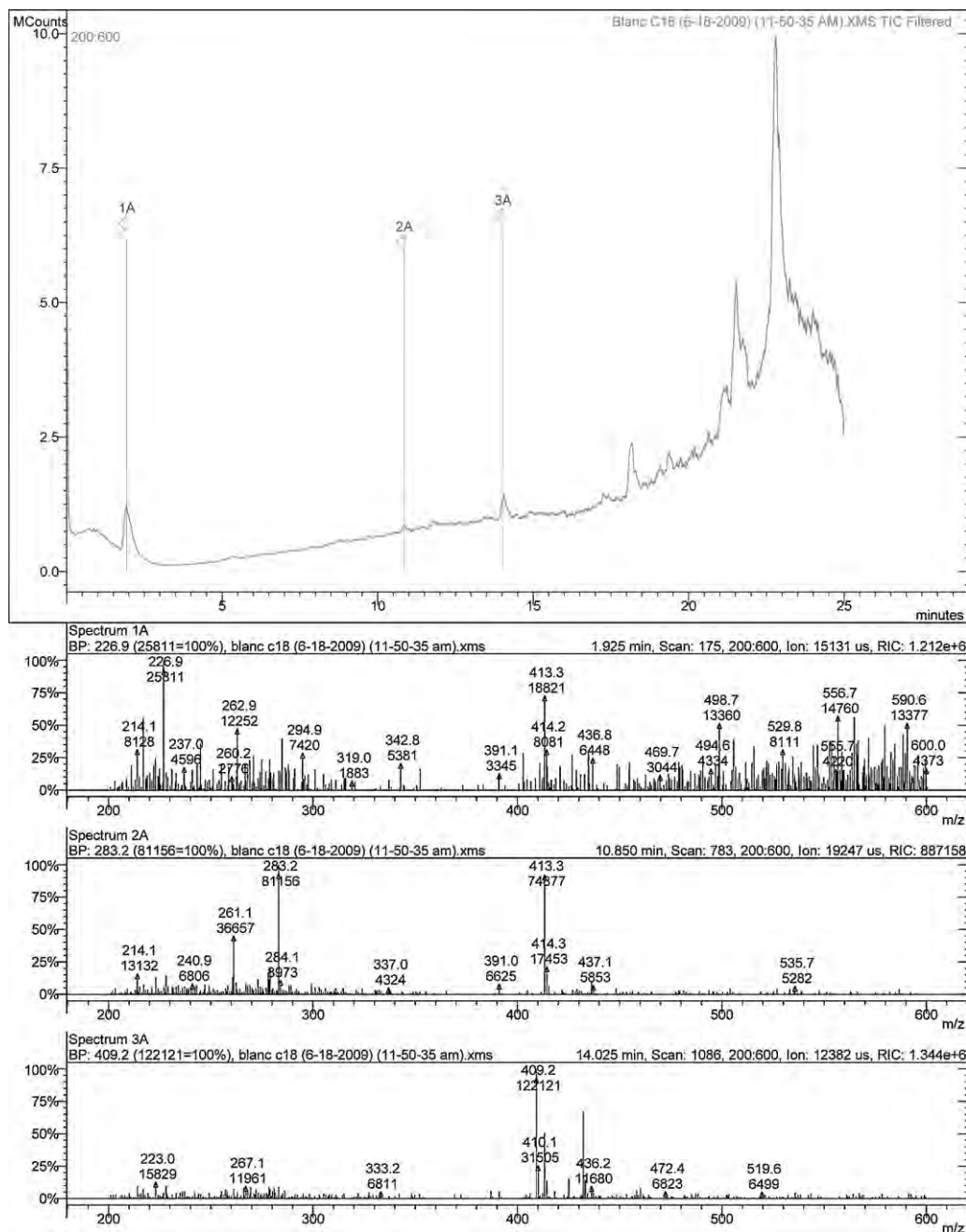
Fig. 6. HPLC–MS/MS of a C18 cartridge plasma extract of a rat receiving a plain oil gavage. This is a blank for Fig. 7. The range of masses analysed was 200–600. Fragmentation spectra for peaks 1–3 are also included.

dence with OE and estrone whatsoever. The more hydrophilic twin peaks could be associated with very low molecular weights when compared with a series of protein standards, 10.6 kDa and <4 kDa. However, these estimations were not fully applicable because the type of column used is not a perfect molecular weight sieve because deep adsorption interactions are also produced with lipophilic compounds. In any case, the most distinct label presence (accounting for more than half of the label in the sample) corresponded to fairly hydrophilic compounds. No ^{14}C label was found associated with OE and only a small part with estrone.

2.4.4. Low OE levels in rat serum

The MS/MS analysis of these peaks (Figs. 6 and 7) did not show any fragment similar to that of estrone or OE (nor any other of the

estrogenic compounds tested). The approximate molecular weights for these peaks were estimated to be at least 2–4 kDa. After MS/MS, their main fragments (parental: secondary) were (595: 533, **288**) and (306: **219**), i.e., they were in part coincident. However, they were different from those obtained for estrone (271: **253**, 156) or OE (535: **271**, **253**). As expected, OE showed the same fragmentation as estrone. Nevertheless, we have no proof that either of these peaks actually corresponded to the labelled estrone fraction compounds, that there was a single compound in each peak or that what we were looking for was sufficiently ionised to be present in the mass spectrograms.

Notwithstanding, we found that the C18 columns were able to retain the label from water-diluted serum; the ^{14}C label was then eluted with methanol (and methanol–water mixtures in propor-
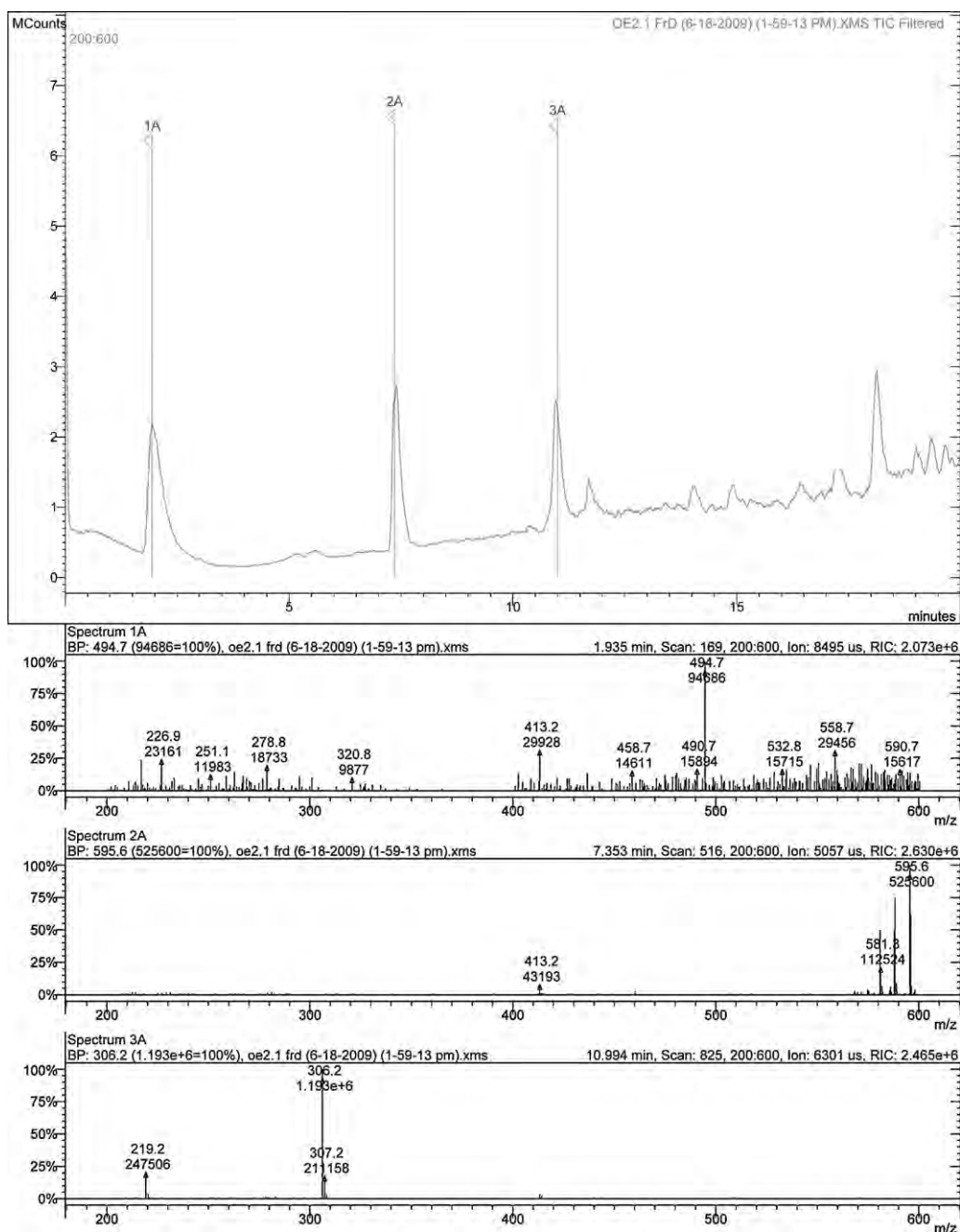
Fig. 7. HPLC–MS/MS of a C18 cartridge plasma extract of a rat receiving an OE gavage 2 h before extraction. The range of masses analysed was 200–600. Fragmentation spectra for peaks 1–3 are also included.

tions up to 1:1), which were conditions that maintained estrone and OE retained in the column. A very approximate calculation of concentrations from the specific activity of the estrone moiety (as administered by the gavage) placed the possible concentration of the labelled compounds in the 0.1 μM range (Table 2). However, precise calculations could not be performed because of the small sample size, low overall label recovery and limited number of samples. We also found that when analysing whole blood, the packed red blood cells retained a significant proportion of the ^{14}C label that was much more than justified by trapped plasma (about 25–30% of all blood label).

There were no significant differences in ^{14}C peak size or recovery between the controls and OE-loaded rats, which may in part be a consequence of the small size of the sample (4 animals each), the very low recovery of label, the smallness of the peaks and indi-

vidual differences between the rats. The specific activity of sera ranged from 90 to 180 Bq/mL regardless of having received the same gavage dose in terms of kBq/g or the rats having a similar weight.

2.5. Subcellular fraction binding

2.5.1. Nuclear receptor binding of OE-derived label

One approach to clarify the existence of specific OE-derivative receptors that are different from the estrogen receptors is the development of a strategy in which direct binding of the complex OE derivative-receptor to the cell nucleus is analysed.

OE induces marked changes in the expression of a number of enzymes and regulatory proteins controlling the metabolism of energy in liver [19], WAT [18], BAT [17] and other organs, as well as

the metabolism of cholesterol in liver [49] and the metabolism of steroid hormones in liver, testicle, adrenals and other organs [50]. WAT is a good target because of the deep changes it experiences under OE treatment [51], but the large mass of fat and the diversity of metabolic activity at its sites [52] make the standardisation of systematic studies with cell organelles difficult. In contrast, liver is a recurrent target for OE action in a number of diverse mechanisms and is usually the tissue of choice for the isolation of cells and its organelles because liver has a robust ability to withstand in vitro manipulation. We decided to use liver cell fractions to further investigate the mechanism of action of the plasma-carried OE-derived metabolite, which we considered to be the compound responsible for the OE-induced metabolic changes.

The standard preparation consisted of three fractions: (a) serum, obtained from rats receiving a ^{3}H-OE gavage as described above; (b) nuclei, isolated from otherwise untouched rats; and (c) cytosol from the same untouched rats, as well as cytosol from the liver cells of the rats receiving the ^{3}H-OE gavage.

After the rats were sacrificed, their livers were excised and cut in rough large pieces that were kept in chilled Krebs–Ringer bicarbonate buffer supplemented with 0.3 M sucrose [53]. A portion of the fragments were taken out and homogenised in 5 volumes of HED buffer (25 mM HEPES, 19 nM NaCl, 1 mM dithiothreitol, 1 mM EDTA) in the cold, using a teflon/glass Potter–Elvejhem manual homogeniser, and centrifuged for 7 min at $1000 \times g$. The precipitate was centrifuged in 2.3 M sucrose for 1 h at $50,000 \times g$ to obtain a clean nuclear pellet. The supernatant of the first low speed centrifugation was again centrifuged for 1 h at $200,000 \times g$ to obtain a cytosol fraction free of organelles. Other samples were homogenised with several strokes in an old and worn Potter–Elvejhem manual homogeniser. The homogenate was roughly filtered through a double layer of hose and centrifuged for 5 min at $100 \times g$, and the nuclear precipitate was washed twice with buffer and inspected for integrity and homogeneity under the microscope. The nuclei were gently suspended in buffer and kept on ice until needed.

Fresh nuclei were incubated with cytosol from ^{3}H-OE-laden rats (receiving a single 10 nmol OE dose, or 1.7 MBq ^{3}H-OH) for 0 or 120 min at 37 °C in a slow moving shaking bath. The final volume contained 2 mL of nuclei suspension (about 0.8 mg protein), 15 mL of cytosol extract (about 225 mg protein, which was equivalent to the total liver aqueous fraction diluted 1:1 with buffer). The control (time 0) was immediately chilled and centrifuged (30 min at $200,000 \times g$) to separate the nuclear and cytosol fractions; 120 min later, the second tube was also chilled in ice and centrifuged. The nuclei were washed once with 1 mL buffer and then counted. Typically, time 0 displayed background counts, but after 60 min, a small amount of label was found attached to the nuclei (in the range of 2–3% of the cytosol label, amounting to a few hundreds of Bq). Further washing of the nuclei with buffer did not result in a significant loss of the label. However, treatment of the nuclei with DNase (Sigma) solubilised the label, which was then lost on washing.

Using clean (i.e., not labelled) cytosol laden with ^{3}H-estrone or estradiol, the binding of label to the nuclei was high. However, the addition of either estrone or estradiol to cytosols from rats receiving a tritium-labelled OE gavage did not hamper the binding of the label by the nuclei; these hormones were able to bind to the estrogen receptor, but did not interfere with the binding of the labelled OE-derivative to nuclear DNA because they bound to different receptor sites in the nucleus.

2.5.2. In vitro exposure of nuclear receptors to labelled OE

Albumin (fatty acid-free, from Sigma) was dissolved in Krebs–Ringer bicarbonate buffer (1 mL, 35 g/L) and incubated at room temperature under gentle shaking for 12 h with 5 M OE (12 kBq of ^{3}H-OE) added in 0.1 mL of methanol under vigorous

shaking to better distribute the microprecipitate. The albumin suspension was cleaned by passing once through a C18 cartridge (SPE-C18), which retained most of the unattached OE. The specific activity of labelled OE-laden albumin was 357 Bq/mg protein.

To 2.5 mL cytosol, 1 mL of OE-laden albumin was added and incubated at 37 °C for 1 h. Separation of albumin with a Blue Sepharose column (Pharmacia) resulted in the retention of 82% of the label in the albumin, with the rest being transferred to other cytosol proteins. Incubation of fresh nuclei with this labelled cytosol, however, resulted in practically no binding of label to the nuclei, both in time 0 controls and after incubation. Albumin-OE also did not interfere with the binding of estrone and estradiol when following the protocol indicated above. The elimination of cytosol albumin using Blue Sepharose columns did not retain the cytosol label, which indicates that the OE-derived label in liver cytosol was not bound to albumin.

These data showed that the OE-derivative was bound specifically by the nuclei. Therefore, there was a nuclear receptor or a cytosolic receptor that was able to be transposed to the nucleus. This binding occurred at a site different from the estrogen receptor(s) to which estradiol and estrone bind, and thus did not interfere. OE did not bind the estrone receptor when incubated with nuclei, a fact that was already tested by us when we found that OE did not bind to the estrogen receptor β [54].

3. Discussion and conclusions

3.1. Differentiation of the effects of OE as pharmacological agent from those of its derivative W

In spite of OE being a normal component of plasma, the variability of its levels and their low entity compared with W suggest that this is not the real metabolic signal controlling body fat size. This role is probably played by OE's derivative, W. The estimated levels of W change in accordance with the metabolic effects sought by OE administration.

The nature of the OE-derivative, however, has not yet been elucidated. We were unable to clarify the nature of the OE-derivative because we painstakingly followed a number of false leads, based essentially on the "facts".

(a) OE administration results in a repetitive, dose-dependent chain of effects deeply altering the metabolism and energy homeostasis [2,55].

(b) The administration of the estrone moiety alone produces, at low concentration, an effect opposite to that of OE [26]. OE at low concentrations has the same effect as higher concentration, but the effects are less marked [56].

(c) β-Estradiol administration slightly lowers body weight [57–59], but not in the same form, intensity and dose-dependent manner as OE [2]. OE also inhibits the formation of estradiol by inhibiting estrone 17β oxidation [50].

(d) OE has no marked estrogenic effects (as those of β-estradiol) when given orally [53], but produces hypoandrogenicity instead [50].

(e) The OE label finds its way easily into the blood and tissues [33], which is considered proof that it is actually taken up.

(f) "Acyl-estrone" can be measured in plasma with specific antibodies for estrone [25]. Therefore, the final derivative must travel in plasma in a form in which the estrone nucleus is recognisable.

(g) Estrone is synthesised by adipose tissue in significant amounts [60]; it is excreted essentially as estrone and a number of its hydroxylated derivatives [61,62]. The excretion of estrone is so massive and the molecule is so resistant that it remains in the

soil and sedimentary strata for long time [63]. Most of the estrogens in the market come from conjugates and other derivatives in the urine of pregnant mares [64].

Of these "facts" only (e) and (f) were not sufficiently proven. In this study, we have found that they were not completely true. However, the remaining "facts" stand. Substitution of estrone by estradiol or other estrogens does not elicit the same results [29]. Thus, OE is effective as a drug against obesity, and OE or W is present in the body in amounts related to the mass of fat reserves [36,65]. However, OE does not travel in plasma in significant amounts [66], as found here. Instead, we found a more hydrophilic derivative that binds liver nuclei and travels in plasma, but whose structure is largely unknown. We named this compound "W". We can only speculate about its concentrations from our early works using a rather non-specific antibody. We have not been able to prove that it contains an intact estrone nucleus. If it does not, then why is so much estrone produced and excreted? Why is so much structural specificity needed for OE to act as a drug if not as donor of the estrone nucleus in a form that is not directly accessible by the free hormone?

The limited, but constant, presence of real OE in plasma is consequent with this molecule being the actual precursor of W, in a way that is similar to its formation from OE gavages, but not directly from estrone administrations. The WAT synthesis of OE, its presence in this tissue and in plasma lipoproteins and its role as W's precursor suggest that the making of a WAT tissue mass-dependent ponderostat signal based on estrone derivatives is a much more complex affair than previously assumed.

3.2. Postulated nature of the functional OE derivative (W)

The relative hydrophilia of the functional OE derivative points suggests that estrone is not part of the molecule because the estrone nucleus is extremely hydrophobic and resistant to chemical modification. However, we did not find free estrone fragments in the MS/MS breakup of the purported W peak. Thus, we can assume that the modification of the estrone nucleus is done through the most probable (and easy) way, the hydroxyl in C3. Consequently, the path synthesising W from OE requires the displacement/substitute of the oleoyl moiety. Perhaps this is the reason why OE is a good precursor of W and free estrone (and other esters) is not. In any case, if estrone is part of the W molecule (as we assume), it needs a fairly hydrophilic attachment to compensate for its marked hydrophobicity. The retention of this compound in C18 columns suggests that the derivative is probably a polarised molecule, with a hydrophobic region (more or less transformed estrone nucleus) and a hydrophilic side chain, probably bound on C3.

Saponification does not free MS/MS-identifiable estrone. Thus, either the estrone is modified in a way that results in it being unrecognisable to the MS system using the limited methodology available, or it is bound to the hydrophilic attachment in a way that is not susceptible to breakage by saponification and is insensitive to acyl-cholesterol esterases that easily hydrolyse OE. Alternatively, at least, W is not broken up by these procedures to yield free estrone. We can infer that the side chain attachment to estrone is probably not an ester bond.

The molecular weight of W is relatively low and is probably lower than OE. However, we cannot speculate further with the dearth of information (and accumulated disinformation) on the nature of this compound.

Thus, we can only conclude that:

(a) There is an active OE "derivative", W, whose complete structure we do not know but that necessarily has to maintain some rela-

tionship with the original OE molecule (i.e., at least containing the estrone nucleus) because of structural constrictions and the above cited general considerations on the role of estrone.

(b) This compound must necessarily travel in the bloodstream to reach its target organs. Consequently, its concentration must change with higher loads of the precursor OE. Alternatively, its levels should parallel the effects observed. Because OE effects are dose-dependent, those of the derivative must also be dose-dependent. The changes agree with those of plasma label after tritium water is discounted.

(c) W belongs to a new class of hormonal signals (of which none have been described so far), which are derived from steroidal hormones, and has nuclear receptors different from the estrogen receptors.

3.3. Perspectives

OE was developed as an anti-obesity drug. The treatment of a severely morbid obese human with OE showed dramatic slimming effects with no secondary effects, nor changes in his plasma chemistry or steroid hormone levels [67]. OE was later subjected to more orthodox clinical trials, with excellent phase I results; however, phase II did not show sufficiently significant losses of weight in the experimental subjects. For this reason, the development of OE as a slimming drug was discontinued, in spite of the results being in the expected range for the doses given [68]. In spite of this drawback, investigations to understand how OE induced these peculiar effects on energy partition have continued. The data presented here summarise the latest efforts to characterise W, the active form of OE. The eventual uncovering and administration of W (instead of OE) as an anti-obesity (and anti-diabetic hypocholesterolemic) drug may retain the powerful positive effects of OE without its inconvenient loading of estrone and negative effects on testosterone metabolism. We continue working along this line to establish the complete chemical nature of this singular compound. Although it is obscured by many other plasma components, this is an elusive albeit very important target.

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