Investigation of the *in vitro* metabolism of 17β-estradiol by LC-MS/MS using ESI and APCI

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Liquid chromatography coupled to tandem mass spectrometry with electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) were used for the investigation of the *in vitro* metabolism of 17β-estradiol on rat isolated hepatocytes. The catechol estrogens corresponding to 17β-estradiol and estrone were identified by ESI, together with their glutathione and glucuronide conjugates, whereas more apolar compounds such as methoxylated catechol estrogens were characterised using APCI.

Introduction

The steroid hormone estradiol is mainly metabolised (i) by oxidation of the hydroxyl function at the C17 position *via* steroid dehydrogenase, leading to estrone, and (ii) by hydroxylation via cytochrome P450 enzymes, occurring on the preferred positions C16α, C2 and C4 of the steroid (Fig. 1). When the hydroxylation takes place on the aromatic A ring of the steroid (positions 2 and 4), it leads to the formation of catechol-estrogens [1]. These compounds undergo several enzymatic processes to be either converted into inactive O-methoxylated metabolites or oxidised into quinone forms. Due to their highly electrophilic properties, estrogen quinones are extremely reactive species. Although they can be inactivated by conjugation with glutathione [2], they are also likely to react with DNA bases to form covalent adducts. This process can induce mutagenicity phenomenons [3], and is known to constitute a critical first step in several tumor initiation processes.

The aim of this work was to study the *in vitro* metabolism of 17β-estradiol using hepatocytes in order to assess the formation of catechol estrogens at the cellular level. Thus, the detection of the catechol estrogens and their subsequent biotransformation products, *i.e.* glucuronide or glutathione conjugates and O-methoxylated derivatives, has been particularly emphazised. The extracts obtained from rat hepatocyte incubations have been directly analysed by LC-MS/MS using ESI and APCI ionisation techniques, which proved to be very complementary for the determination of polar as well as non-polar metabolites.

Material and methods

Chemicals

[^{14}C]Estradiol-17β (99 % HPLC radiopurity) and unlabelled estradiol were respectively purchased from NEN (Les Ulis, France) and Sigma (L'Isle-d'Abeau-Chesnes, France). Standard estradiol glucuronides were obtained from Sigma. Standard reference compounds of the various catechol estrogens, their glutathione conjugates and their methoxylated derivatives have been synthesised according to previously published procedures [4,5,6,7]. HPLC grade solvents from Scharlau (Scharlau, Barcelona, Spain) and water from a Milli-Q system (Millipore, Saint-Quentin-en-Yveline, France) were used for LC-MS analyses.

Cell preparation

Hepatocytes were prepared from Wistar rat livers according to the method described by Seglen [8]. The viability of the

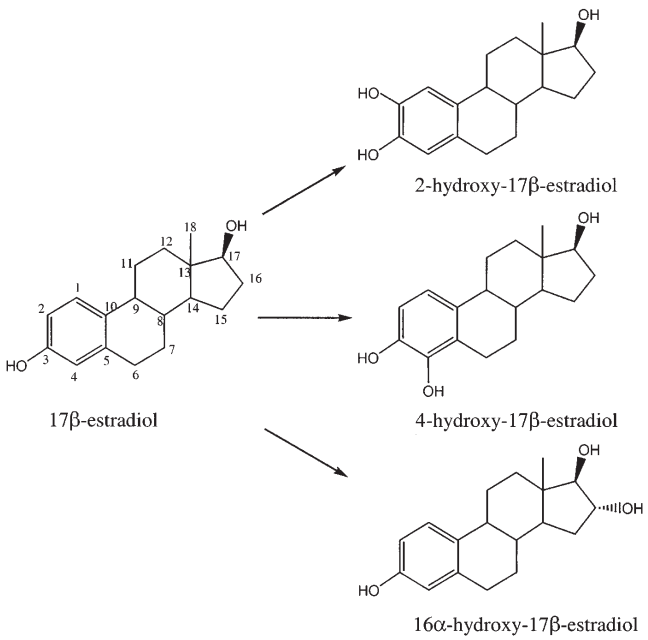

Figure 1. Structures of estradiol and major hydroxylated metabolites.

Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:2000280273

cells was 79.5 % as measured by the trypan blue exclusion test. The hepatocytes were diluted in MEM (Minimum Essential Medium Eagle) to give a final concentration of 3×10^6 cells/ml.

Incubations

Incubations with isolated hepatocytes (final volume 3 ml) were carried out under air at 37 °C in six-well plates (Nunclon, Roskilde, Denmark) with constant shaking. After a 5 min preincubation period, [^{14}C]estradiol (specific activity of 11.5 MBq/mmol) was added in 5 µl of ethanol, at a concentration of 250 µM for incubation. After 4 h, 0.5 ml of ascorbic acid (5 % in water) was added to the medium and the supernatants were removed and frozen before analysis.

LC-MS analyses

LC-MS analyses were carried out using a Thermo Separation P4000 pump (ThermoQuest, Les Ulis, France) fitted with a Rheodyne 7725i injector. UV detection was achieved at 280 nm using a Thermo Separation UV1000 detector. Different chromatographic systems were used depending on the ionisation technique required for LC-MS. The mass spectrometry analyses were carried out on a Finnigan LCQ quadrupole ion trap mass spectrometer equipped either with an electrospray or an APCI source. All analyses were performed under automatic gain control conditions, using helium as damping as well as collision gas for MS/MS experiments.

System I: LC-ESI-MS

The column was an Uptisphere ODS-B (250 × 4.6 mm I.D., 5 µm) from Interchim (Montluçon, France). The mobile phases consisted of water/acetonitrile/acetic acid mixtures, 95:5:0.5 for A and 45:55:0.5 for B. The following gradient was used: 0-5 min, 100 % A; 5-10 min, linear gradient from 100 % to 80 % A; 10-15 min, 80 % A, 15-50 min, linear gradient from 80 % to 40 % A; 50-65 min, 40 % A; 65-75 min, linear gradient from 40 % A to 100 % B, and then 75-90 min, 100 % B. The flow rate was 1.5 ml/min and a post-column splitting was achieved in order to ensure a 300 µl/min flow rate entering the electrospray ionisation source. The electrospray needle voltage and heated capillary temperature were set to 5.0 kV and 230 °C, respectively. The heated capillary voltage was – 16 V and the tube lens offset was 0 V.

System II: LC-APCI-MS

The column was a Lichrosorb C18 (250 × 4.6 mm I.D., 5 µm) from Merck (Nogent-sur-Marne, France). The mobile phases were water/methanol mixtures, 75:25 for A and 10:90 for B. The following gradient was used: 0-15 min, linear gradient from 100 % to 46 % A; 15-30 min, 46 % A, 30-45 min, linear gradient from 46 % A to 100 % B; and then 45-75 min, 100 % B. The flow rate was 1 ml/min without splitting into the ionisation source. The APCI source was used with a nebuliser temperature of 450 °C and a discharge current of 5 µA. The heated capillary temperature was set to 150 °C. The voltages of the heated capillary and the tube lens were – 5 V and – 35 V, respectively.

Results and discussion

The radio-chromatogram obtained from the injection of the supernatant of the isolated hepatocytes incubated with 17β-estradiol is presented in figure 2a. It exhibits at least 17 peaks corresponding to *in vitro* metabolites of 17β-estradiol. As indicated by the arrow placed in figure 2a at the retention time of 17β-estradiol (**15**), the parent compound has been quickly metabolised within one hour of incubation. Representative reconstructed ion chromatograms from the LC-MS analysis of the same sample using negative electrospray ionisation are reported in figure 2b. Due to separated injections on different systems, little discrepancies were observed between the retention times obtained on the radio-HPLC and LC-ESI-MS experiments. Nevertheless, each peak of the radioactive trace could be detected and identified in the LC-MS experiment using negative electrospray ionisation, except for the peaks eluted at the end of the gradient elution (*ca*. 60-70 min), corresponding to the relatively non-polar metabolites for which the electrospray ionisation does not seem to be appropriated. These compounds were analysed using negative atmospheric pressure chemical ionisation (APCI) with a different chromatographic system, and will be treated at the end of this section. A comprehensive view of the identified metabolites and their quantitative distribution (expressed in percentage of the detected radioactivity) is given in table I.

Hydroxylated metabolites

Compound **1** eluted at 27.9 min and exhibited a $[M-H]^-$ ion at m/z 303. This value is consistent with a bis-hydroxylated derivative of estradiol. Further investigations using MS/MS experiments are now in progress in order to establish the formal identity of this compound. However, taking into account the main biological hydroxylation processes of estrogens [1], the most probable structure for **1** may be the 2,16α-dihydroxy-estradiol.

Hydroxylated forms of estradiol and estrone accounted for 8.2 % and 10.7 % of the total detected radioactivity, respectively (Tab. I). They were detected by LC-ESI-MS at m/z 287 and m/z 285, respectively, at retention times of 51.2 min and 56.7 min (**13** and **14**, figure 2). These m/z values were consistent with $[M-H]^-$ ions for catechol estrogens. This was confirmed from the acquisition of the corresponding MS/MS spectra of the $[M-H]^-$ ions generated using APCI (data not shown). Furthermore, the fragmentation pattern of both compounds using collisionally activated decomposition (CAD) into the ion trap device could be used for the determination of the hydroxylated position of the 17β-estradiol. Comparison of the retention times and MS/MS spectra of **13** and **14** with those obtained from standard reference compounds (2-hydroxy-estradiol, 4-hydroxy-estradiol,

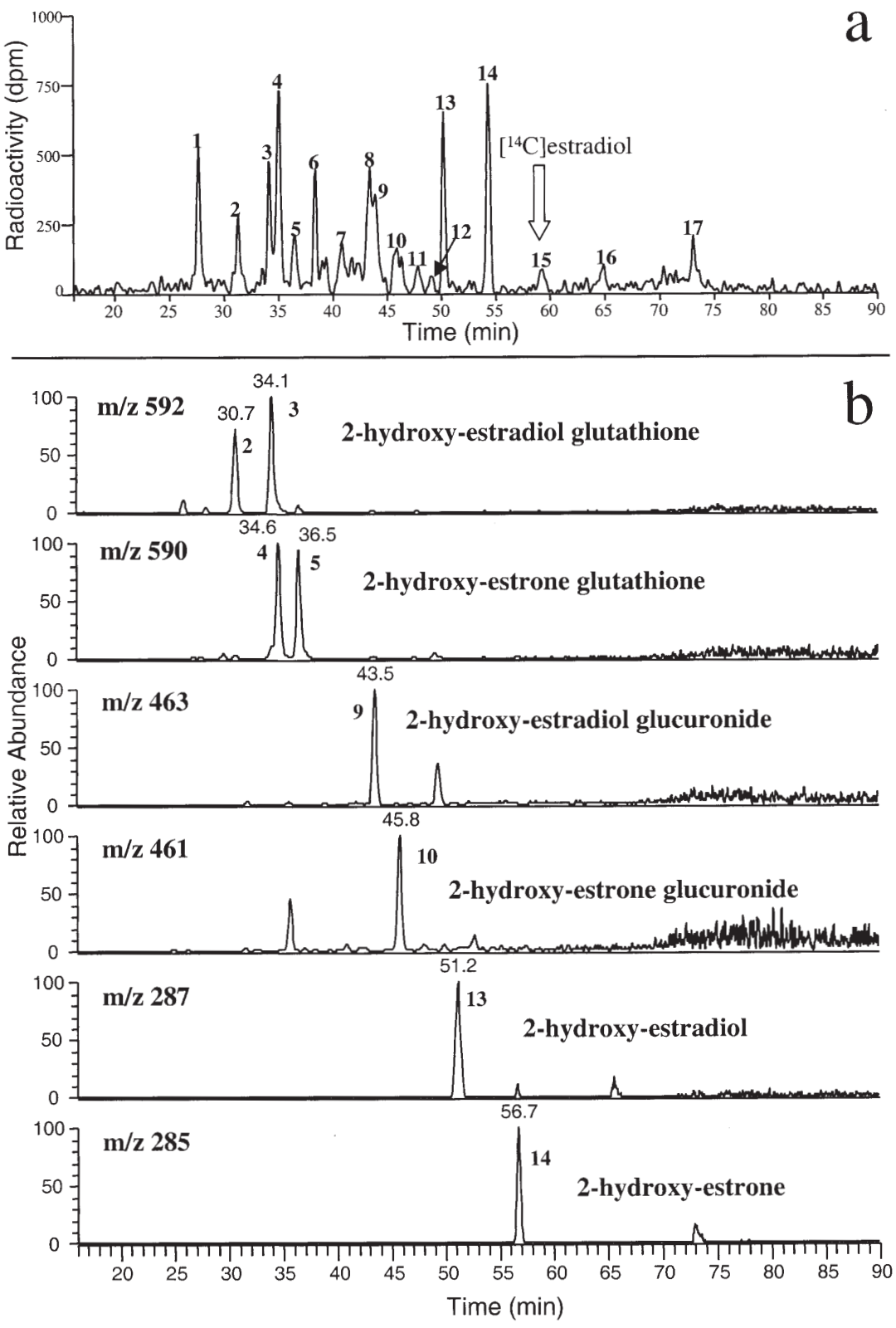

Figure 2. Chromatograms obtained by LC analysis of the hepatocyte culture supernatant. (a) Radio-HPLC chromatogram and (b) LC-ESI-MS chromatograms (traces represent extracted currents for the $[M-H]^-$ ions of representative metabolites at m/z 592, 590, 463, 461, 287 and 285).

Table I. 17β-estradiol metabolites identified in the LC-MS analysis of hepatocytes incubation supernatants. Percentages were determined by integration of each radioactive peak in the profile. Fragment ions marked with an asterisk were obtained from $[M-H]^-$ ions generated by APCI (#: tentatively identified; *n.d.*: not determined).

Peak number	*%*	*Compound*	*$[M-H]^-$, m/z*	*Main characteristic fragment ions, m/z*
1	7.5	2,16α-dihydroxy-estradiol[#]	303	*n.d.*
2	3.5	1-S-glutathione-2-hydroxy-estradiol	592	463, 319, 306, 272, 254, 179
3	6.9	4-S-glutathione-2-hydroxy-estradiol	592	463, 319, 306, 272, 254, 179
4	11.4	1-S-glutathione-2-hydroxy-estrone	590	461, 317, 306, 272, 254, 179
5	3.8	4-S-glutathione-2-hydroxy-estrone	590	461, 317, 306, 272, 254, 179
6	5.3	estradiol-3-glucuronide	447	429,175
7	4.4	-	-	-
8	8.2	estradiol-17-glucuronide	447	429,175
9	6.9	2-hydroxy-estradiol-glucuronide	463	445, 401, 287, 175
10	3.3	2-hydroxy-estrone-glucuronide	461	443, 399, 285, 175
11	2.1	2-methoxy-estradiol-glucuronide	477	459, 417, 301, 175
12	1.0	2-methoxy-estrone-glucuronide	475	457, 415, 299, 175
13	8.2	2-hydroxy-estradiol	287	269, 162*
14	10.7	2-hydroxy-estrone		285 269, 161*
15	1.7	estradiol	271	253, 183, 145
16	2.4	2-methoxy-estradiol	301	286*
17	3.2	2-methoxy-estrone	299	284*

2-hydroxy-estrone, 4-hydroxy-estrone) allowed to identify the catechol estrogens detected as the 2-hydroxy-estradiol and 2-hydroxy-estrone. The radioactive trace (Fig. 2a) shows that these two compounds represent the main metabolites formed after 1 h incubation of 17β-estradiol with rat liver hepatocytes. Thus, it is concluded that the main enzymatic hydroxylation process on the aromatic A ring occurred on the position C2 of estradiol as previously described on rat liver micosomes [9], and that no hydroxylation at the position C4 of the steroid was observed.

Glucuronic acid conjugates

The occurrence of glucuronic acid conjugates of estradiol could be evidenced by their corresponding $[M-H]^-$ ions at m/z 447. Indeed, two distinct isomeric glucuronides were detected (**6** and **8**, Fig. 2a). By comparison of their MS/MS spectra with those obtained from standard reference compounds [10], **6** is assigned to the estradiol-3-glucuronide and **8** to the estradiol-17-glucuronide.

Glucuronic acid conjugates of hydroxy-estradiol and hydroxy-estrone were also detected as their $[M-H]^-$ ions at m/z 463 and 461, respectively (**9** and **10**, Fig. 2). Their corresponding MS/MS spectra exhibited the characteristic pair of complementary fragment ions at m/z 175 and m/z 287 (**9**, Fig. 3a) or m/z 285 (**10**, Fig. 3b). The origin of these fragment ions can be explained from the scission of the glycosidic bond between the steroid and the glucuronic acid moiety. The m/z 175 and $[M-H-176]^-$ ions can be considered as characteristic of glucuronide conjugates as they appear on most of MS/MS spectra obtained from such compounds [10,11,12]. Since the hydroxylation of the estrogens occurred at position C2 of the steroid as indicated by the structure of **13** and **14**, the glucuronic acid conjugates evidenced herein very likely correspond to 2-hydroxy-estradiol-glucuronide and 2-hydroxy-estrone-glucuronide, respectively. Indeed, the glucuronic acid can be linked either to the 17β-hydroxy or to the 3-hydroxy groups. However, no complementary work could be carried out in the absence of the corresponding standard reference compounds.

In the same manner, two additional minor glucuronic acid conjugates could be evidenced, corresponding to **11** and **12** (Fig. 2a) and accounting respectively for 2.1 % and 1.0 % of the radioactivity. The $[M-H]^-$ ion of these species were located at m/z 477 and m/z 475, for **11** and **12**, respectively. These values represented a 14 mass units shift in comparison with **9** and **10**, which can be attributed to a metabolic methylation process. Moreover, as in the case of **6**, **8**, **9** and **10**, the presence of the m/z 175 and $[M-H-176]^-$ ions on the MS/MS spectra was considered as diagnostic for the identification of glucuronides (Tab. I). As a consequence, **11** and **12** could be considered as the O-methylated derivatives of **9** and **10**, and were identified respectively as the glucuronic acid conjugates of the 2-methoxy-estradiol and 2-methoxy-estrone.

Glutathione conjugates

Compounds **2** and **3**, eluted at 30.7 and 34.1 min, respectively, both exhibited quasi-molecular $[M-H]^-$ ions at m/z 592, whereas **4** and **5** (34.6 and 36.5 min) were detected at m/z 590. Their quantitative determination is reported in

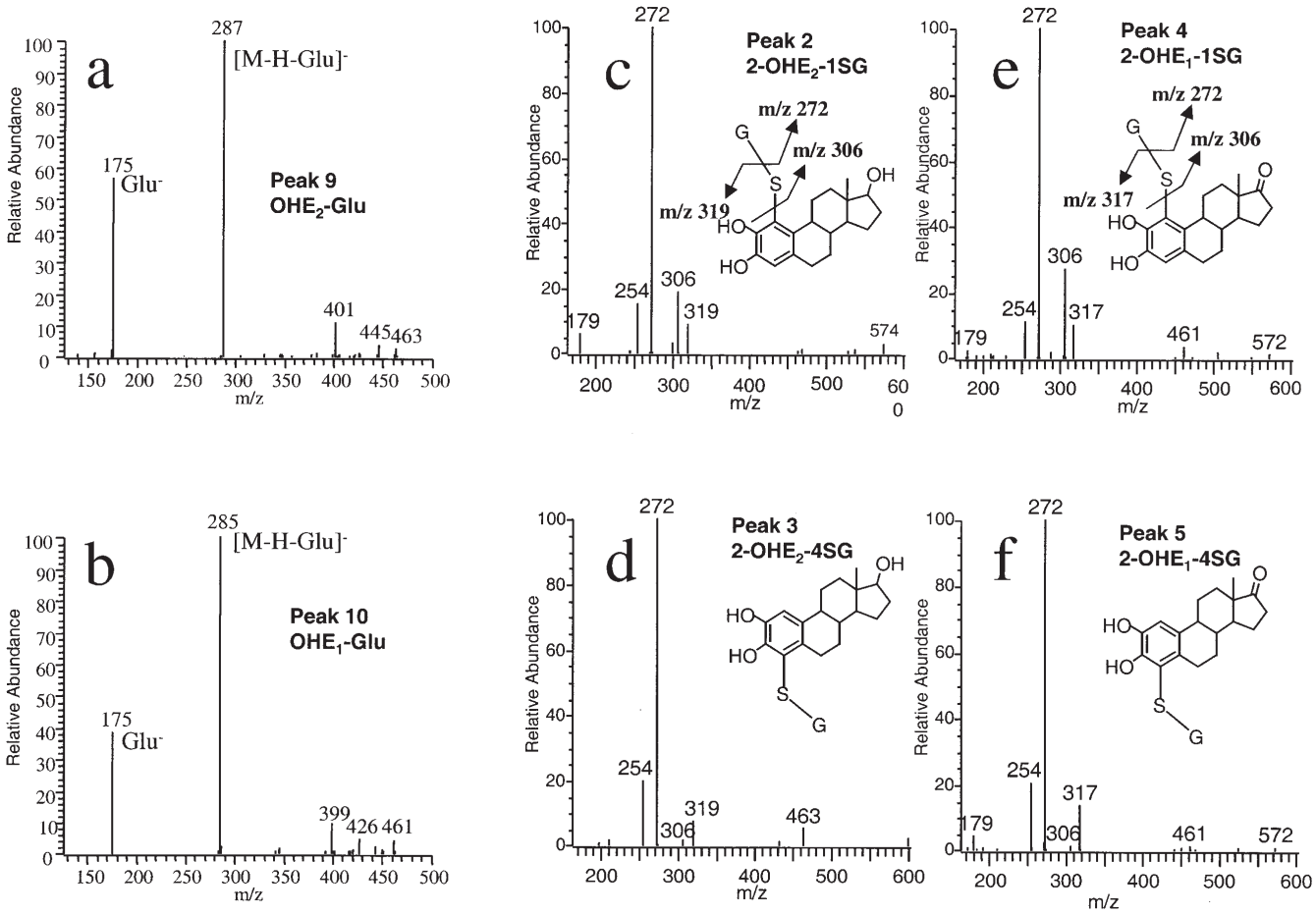

Figure 3. Product-ion spectra from collisionally activated decompositions of ESI produced [M-H]⁻ ions. (a) m/z 463 ion from 9 and (b) m/z 461 from 10, (c) and (d) m/z 592 ions from 2 and 3, (e) and (f) m/z 590 ions from 4 and 5.

table I. These data are consistent with glutathione conjugates of hydroxylated estradiol and estrone, respectively. The MS/MS spectra of the $[M-H]^-$ ions for **2-3** and **4-5** displayed very similar fragmentation patterns (Fig. 3). In particular, the base peak of the MS/MS spectra is observed at m/z 272 whatever the compound considered. This indicates that this fragment ion is related to the glutathione part rather than the estrogen part of the molecule. The same observation can be done for the m/z 254 ion, very likely originating from a consecutive dehydration of the m/z 272 ion. As a matter of fact, the main decomposition pathways of the $[M-H]^-$ parent ions under low energy collisional activation consist of the cleavages of both thioether bonds of the glutathione conjugates. Contrary to the corresponding MH^+ ions [13], virtually no peptide bond fragmentation is observed, except for the low abundance m/z 463 (or 461) ion which may arise from the loss of the glutathione glutamic acid residue from the selected $[M-H]^-$ parent ion.

The formation of the two complementary fragment ions at m/z 272 and 319 (or 317) may involve an ion-dipole complex intermediate resulting from the cleavage of the C-S bond of the glutathione cystein moiety. The decomposition of this complex could give rise to the formation of either the m/z 272 or m/z 319 daughter ion by reversible proton migration, depending on the gas phase acidity of the corresponding neutral of such a complex, as already reported for other steroid derivatives [14,15]. A fragmentation mechanism involving such an anion-dipole complex intermediate is proposed in figure 4. According to this scheme, the deprotonation of the glutathione conjugate at the acidic function of its glycine residue can induce the decomposition of the $[M-H]^-$ ion according to an assisted process, leading to the breakage of the CH_2-S bond of the glutathione moiety. The ion-dipole complex thus generated (form a, Fig. 4) can directly decompose into the m/z 319 daughter ion or isomerise into form b (Fig. 4) by reversible proton transfer. The decomposition of this later form (form b) yields the m/z 272 fragment ion. However, no additional experiment has been carried out to further investigate this fragmentation mechanism.

On the other hand, the breaking of the steroid – glutathione linkage leads to the charge retention on the sulfur

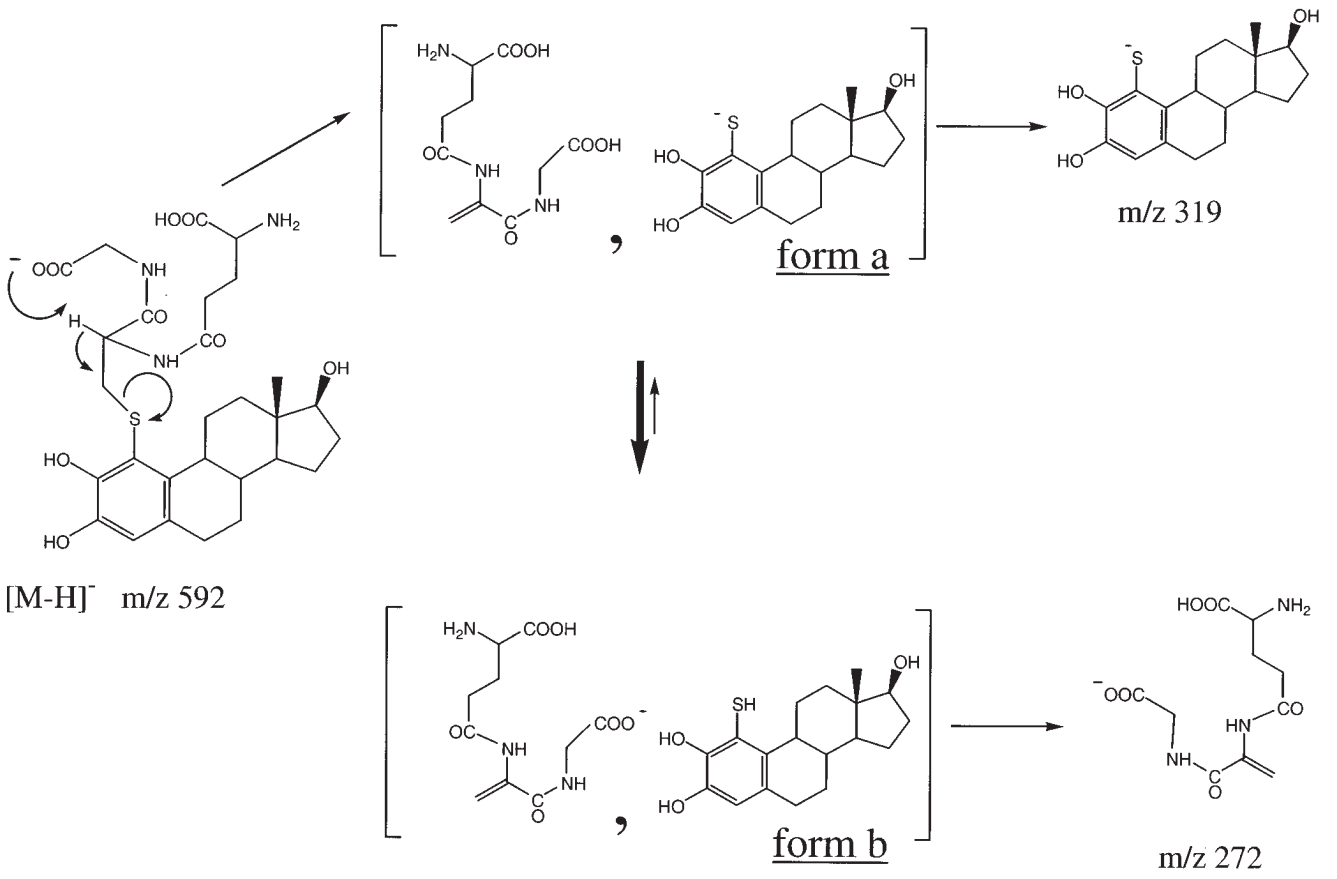

Figure 4. Decomposition mechanism involving an ion-dipole complex intermediate proposed for the formation of the two complementary fragment ions m/z 272 and m/z 319 from the [M-H]⁻ m/z 592 parent ion of compound 2.

atom of the tripeptide glutathione moiety, giving rise to the m/z 306 fragment ion as indicated in figure 3. Glutathione conjugates of estradiol and estrone have been extensively studied by tandem mass spectrometry using positive electrospray ionisation [5,13]. Under the low energy CAD conditions used in the ion trap mass spectrometer, isomeric discrimination was rather difficult and was based only on low abundance fragment ions. In our case, working on deprotonated species, the isomeric glutathione adducts can be easily distinguished from their m/z 319/306 (for estradiol adducts) or m/z 317/306 (for estrone adducts) abundance ratios as they appear reversed in figure 3, depending on the isomer considered. Further investigations are now in progress in order to investigate the fragmentation mechanisms of the various isomers using standard reference compounds. Nevertheless, in the work presented herein, the identification of the glutathione conjugates could be achieved using coinjection experiments with the standard derivatives. Thus, all the glutathione conjugates characterised in this work could be identified as 2-OH estrogen derivatives on the basis of their retention times. Indeed, the conjugation of glutathione with 4-hydroxy estrogens is known to lead to adducts in position C2 whereas the reaction with 2-hydroxy estrogens leads to a mixture of C1 and C4 conjugated compounds [2,5,12]. Injection of our synthesised reference compounds as well as previous results published by the groups of Cavalieri [2] and Bolton [16] indicate that adducts with glutathione linked at position C1 of the A-ring of the estrogen are eluted before their isomers with the attachment of glutathione at position C4. Thus, the following structures could be attributed to the different glutathione conjugates generated *in vitro*: **2**: 2-hydroxy-estradiol-1-S-glutathione; **3**: 2-hydroxy-estradiol-4-S-glutathione; **4**: 2-hydroxy-estrone-1-S-glutathione and **5**: 2-hydroxy-estrone-4-S-glutathione.

Unknown metabolite

Despite some information available from the negative electrospray ionisation experiment, the minor compound **7** could not be identified at present. Work is now in progress in order to determine whether the positive electrospray ionisation would give better results or whether APCI should be used for the characterisation of this compound.

Methoxylated catechol derivatives

APCI was used for the analysis of the methoxylated catechol estrogen derivatives as they could not be detected using negative electrospray ionisation. Firstly, attempts were made to carry out the APCI analyses using the same chromatographic system as for the radio-HPLC and LC-ESI-MS experiments. However, poor results were obtained using acetonitrile in preliminary experiments made on standard methoxylated catechol estrogens, and thus, a different chromatographic system containing methanol had to be developed. Actually, likely owing to its lower gas phase acidity [17], methanol gave better results than acetonitrile for the negative APCI analysis of methoxylated catechol estrogens. It should be noted that using the Lichrosorb column with the water-methanol elution system, the profile of the separation obtained was different compared to the Uptisphere ODS/water-acetonitrile system used for ESI. For example, 2-hydroxy estrone eluted before 2-hydroxy estradiol in the APCI system whereas the reverse elution order was obtained with the Uptisphere stationary phase. The CAD product ion spectra obtained from different reference methoxylated catechol estrogens show that the loss of a methyl radical constitutes the only decomposition path observed from the selected $[M\text{-}H]^-$ parent ion, although higher order MS/MS experiments into the trap may produce more comprehensive structural information. The loss of the methyl group is favoured for 4-methoxylated species compared to 2-methoxylated compounds (data not shown). This observation was used to distinguish 2-methoxylated compounds from their 4-methoxylated isomers, and consequently, 2-methoxy-estradiol and 2-methoxy-estrone could be unambiguously identified in the supernatant of the incubations, corresponding to **16** and **17** (Fig. 2a), respectively. This result was in agreement with the nature of the various compounds identified by ESI, which indicated that the enzymatic hydroxylation process occurred at position C2 of the steroid A-ring.

Conclusion

The ESI and APCI ionisation techniques used in this work proved to be very relevant for the direct analysis of biological samples generated by *in vitro* 17β-estradiol metabolism experiments. The occurrence of catechol estrogens as well as their glutathione and glucuronide conjugates in the incubation supernatants could be clearly evidenced using negative electrospray ionisation. The characterisation of the less polar methoxylated derivatives could be achieved using APCI whereas electrospray ionisation gave no response for these compounds. In the case of glucuronide as well as glutathione conjugates, regio-isomeric compounds could be differentiated using MS/MS experiments on $[M\text{-}H]^-$ quasi-molecular ions. The nature of the various metabolites identified in this work confirms the occurrence of a hydroxylation process on the estradiol aromatic A cycle giving rise to catechol estrogens at the cellular level. In the case of rat liver hepatocytes, this enzymatic process produces regiospecifically the 2-hydroxylated derivatives whereas no hydroxylation at position C4 was observed, contrary to what was recently reported in other studies using rat microsomes.

Acknowledgments

This work was supported by the European Community (grant B6/7920/98/000823). The authors wish to thank Isabelle Jouanin for kindly providing the synthetic reference compounds, and Pr. Jean-Claude Tabet for helpful discussions on this work.

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