Analysis of nucleoside-estrogen adducts by LC-ESI-MS-MS†

The Analyst

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Received 2nd July 1998, Accepted 26th October 1998

Liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) has been used for the assessment of the reactivity of estradiol-2,3-quinone towards deoxyribonucleosides in crude reaction mixtures. The use of LC-MS-MS allowed the direct injection of crude reaction mixtures, thus avoiding tedious sample preparation and purification steps before the mass spectrometric analysis of the prepared adducts. Several original (sometimes minor) adducts could be evidenced with deoxyguanosine and a new addition product has been characterized in the reaction of estradiol-2,3-quinone with deoxycytidine. Multisequential MS^n mass spectrometry performed on a quadrupole ion-trap mass spectrometer enabled the discrimination of isomeric adducts present in crude reaction mixtures. These results show that LC-MS-MS is a very powerful analytical tool for the detection and characterization of adducts in crude reaction mixtures.

Introduction

Hydroxylations achieved by cytochrome P450 enzymes represent a major metabolic pathway of estrogens, leading in particular to catechol estrogens (CE), which undergo enzymatic processes to be either inactivated or converted into highly reactive molecules towards essential macromolecules such as DNA.[1] Actually, metabolic activation of CE can generate quinone forms, considered as highly electrophilic species, which are known to covalently bind to DNA bases.[2–5] Nucleophilic attack of the base at various positions of the steroid, leading to a series of different addition products, is one of the most commonly assumed reaction pathways.[3,6] The formed adducts can be considered as estrogen-bound metabolites generated *in vivo*. Moreover, covalent modification of DNA bases is thought to be a crucial initiating event in the chemical carcinogenesis by estrogens, as well as other genotoxic molecules.[3] For this reason, several techniques have been used for the detection of DNA adducts, such as immunoassays, fluorescence assays and ^{32}P-postlabeling, among which the latter has been used in the case of DNA–CE quinone adducts.[7] Mass spectrometric techniques, and particularly tandem mass spectrometry (MS-MS), have emerged as very powerful tools for the structural elucidation as well as for the quantification of the DNA adducts (for review articles, see ref. 6 and 8). After the pioneering work of Biemann and McCloskey[9,10] on nucleosides, early studies were conducted on DNA alkylated adducts using electron ionization (EI) mass spectrometry.[11] The emergence of soft ionization desorption techniques such as laser desorption,[12] fast atom bombardment[13] (FAB) and more recently, matrix assisted laser desorption ionization[14] (MALDI) as well as electrospray ionization[15] (ESI), in conjunction with the development of MS-MS[16] has allowed easier detection of DNA adducts.[6,8] Several ionization modes and MS-MS experiments have been recently compared for the analysis of polycyclic aromatic hydrocarbons–DNA adducts,[17] and in the case of CE quinones–DNA adducts, FAB-MS-MS,[2] MALDI-MS,[3] and ESI-MS[3–5] have been used for structural investigations.

The aim of this work was to develop a powerful and sensitive detection technique in order to assess the chemical reactivity of estradiol-2,3-quinone (E-2,3-Q) towards nucleic acid bases [*i.e.* deoxyguanosine, (dG) and deoxycytidine (dC)], by directly analyzing the crude reaction mixtures. Actually, estrogen-2,3-quinones are reported as species leading only to stable DNA adducts, whereas estrogen-3,4-quinones can lead to additional depurinating DNA adducts.[2,3] The respective occurrence of these two kinds of adducts depends upon which atom of the DNA base is involved in the nucleophilic attack, resulting or not in the destabilization of the glycosidic bond of the deoxyribonucleoside.[2,5] In the few last years, the increasing ease of liquid chromatography-mass spectrometry (LC-MS) coupling *via* atmospheric pressure ionization techniques[18] has made the mass spectrometer a highly sensitive and highly specific detector for HPLC. It is also capable of providing extended structural information on adducts by means of tandem MS experiments,[19–23] especially at the very low levels encountered in biological samples.[24,25] The LC-ESI-MS-MS method developed in this work provides new data on the chemical reactivity of E-2,3-Q towards deoxyribonucleosides. Thus, the formation of depurinating adducts with dG and of a new adduct with dC could be demonstrated.

Experimental

Chemicals and reagents

Chemical reagents used for the syntheses were purchased from Aldrich (Saint Quentin Fallavier, France). Ultrapure water from a Milli-Q system (Millipore, Saint Quentin en Yvelines, France) was used for the preparation of HPLC eluents. All other HPLC solvents were of the highest commercially available grade from Merck (Nogent sur Marne, France).

Syntheses

2-Hydroxy-17β-estradiol was synthesized from commercially available estradiol according to the procedure described by Stubenrauch and Knuppen[26] and was then converted to estradiol-2,3-quinone (1.7 μmol) using the following modified

† Presented at the Third International Symposium on Hormone and Veterinary Drug Residue Analysis, Bruges, Belgium, June 2–5, 1998.

version of the method developed by Abul-Hajj.[27] Activated MnO_2 (0.1 mmol) was added to a stirred solution of 2-hydroxy-17β-estradiol in 2 ml of acetonitrile at −30 °C under a nitrogen atmosphere. The reaction was complete after 15 min. The adducts were then formed by immediately filtering this mixture (dark yellow solution) into a stirred solution of the deoxyribonucleoside (80 μmol) in 4 ml of $CH_3COOH–H_2O$ (1 : 1). After 5 h at room temperature, solvents were removed under reduced pressure and the crude residue was redissolved in a mixture of $CH_3OH–H_2O–CH_3COOH$ (49 : 49 : 2).

LC-MS

LC-MS analyses were performed on a Finnigan LCQ (Thermo Quest, Les Ulis, France) ion trap mass spectrometer fitted with the Finnigan electrospray ionization source. The typical electrospray needle voltage was 5.2 kV and the heated capillary temperature was set at 220 °C. MS^n experiments were carried out with an isolation window width of 2 u using helium as damping and relaxation as well as collision target gas. All analyses were performed under automatic gain control conditions. The HPLC system consisted of a Thermo Separation P2000 (Thermo Quest, Les Ulis, France) LC pump. Injections (10–20 μl of crude reaction mixtures redissolved in solvent A) were achieved using a Thermo Separation AS3000 autosampler. The LC column was an Ultrabase C_{18} (5 μm, 250 × 2 mm) (Life Sciences International, Eragny, France). The flow rate was 0.2 ml min^{-1} without postcolumn splitting into the ESI source. The mobile phases consisted of methanol–water–acetic acid in the following proportions : solvent A, 10 : 90 : 0.2 and solvent B, 90 : 10 : 0.2. The following gradient was used: 0–5 min, linear increase from 0 to 25% B; 5–30 min, linear increase from 25 to 50% B; 30–40 min, linear increase from 50 to 100% B and finally, isocratic step from 40 to 60 min at 100% B. UV detection (280 nm) was achieved with a Thermo Separation UV1000 detector.

Results and discussion

LC-ESI-MS-MS was successfully applied to the characterization of the addition products formed in the different investigated reactions. Five major adducts were evidenced in the reaction between estradiol-2,3-quinone (E-2,3-Q) and dG, some of them representing new compounds. Isomeric compounds could be discriminated by means of MS^2 as well as MS^3 experiments, taking advantage of the features of the ion trap analyzer at very high sensitivity and sufficient resolution. A new adduct has also been characterized with dC, for the first time in these reaction conditions.[2]

Reaction between E-2,3-Q and dG

The reaction of E-2,3-Q with dG yielded a complex mixture of reaction products as shown by the LC-UV chromatogram reported in Fig. 1a. In addition to unreacted dG in excess and other reaction products such as dimeric forms of E-2,3-Q, several major adducts were indicated, which could be divided into two groups. The first group gave signals at *m/z* 554 as MH^+ species (peaks 3, 4, 5, Fig. 1b), and corresponded to the addition of one molecule of dG to E-2,3-Q. The second group displayed MH^+ ions at *m/z* 438 (peaks 1, 2, 6, Fig. 1c), corresponding to adducts in which the deoxyribose moiety has been lost.

In a preliminary work,[28] we have mentioned the occurrence of two M_r 437 adducts in the reaction of E-2,3-Q with dG, for which it was previously reported that only stable adducts (*i.e.* M_r 553 adducts) were formed.[2,3] In the present investigation, the formation of at least three different major M_r 437 adducts is clearly demonstrated (Fig. 1c). Moreover, the use of sequential tandem mass spectrometry allowed the structural discrimination of these adducts. As an example, the collision-induced dissociation (CID) spectra obtained by resonance excitation of the *m/z* 438 ions from peaks 2 and 6 (Fig. 1c) are reported in Fig. 2a and Fig. 2b, respectively. The fragmentations observed for peak 2 (Fig. 2a and Table 1) almost exclusively concerned a dehydration leading to the *m/z* 420 ion (presumably occurring at the C17 position of the steroid), and the cleavage of the steroid-base bond with charge retention on the base moiety, giving rise to the formation of the *m/z* 152 daughter ion. According to considerations on the various sites involved in reactions of dG with chemical carcinogens,[29,30] the N7 nitrogen atom of dG constitutes the only attachment site for which the nucleophilic attack leads to the destabilization of the sugar-base bond. From this, it was deduced that peak 2 was an adduct in which dG was linked *via* its N7 nitrogen atom, very likely to the C6 atom of the steroid since this site represents an activated site for nucleophilic attack on the B cycle of E-2,3-Q in the semi-quinonic form. The fragmentation pathway of the *m/z* 438 ion from peak 1 was identical to that of peak 2 (Table 1), indicating that peaks 1 and 2 corresponded to structurally very closely related

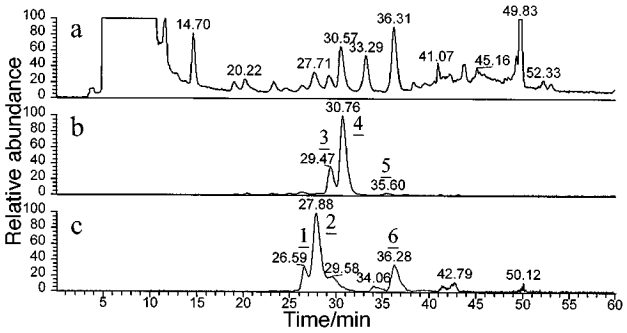

Fig. 1 LC-UV-ESI-MS analysis of an E-2,3-Q–dG crude reaction mixture. a, UV chromatogram (λ = 280 nm) and reconstructed selected ion chromatograms for b *m/z* 554 and c, *m/z* 438.

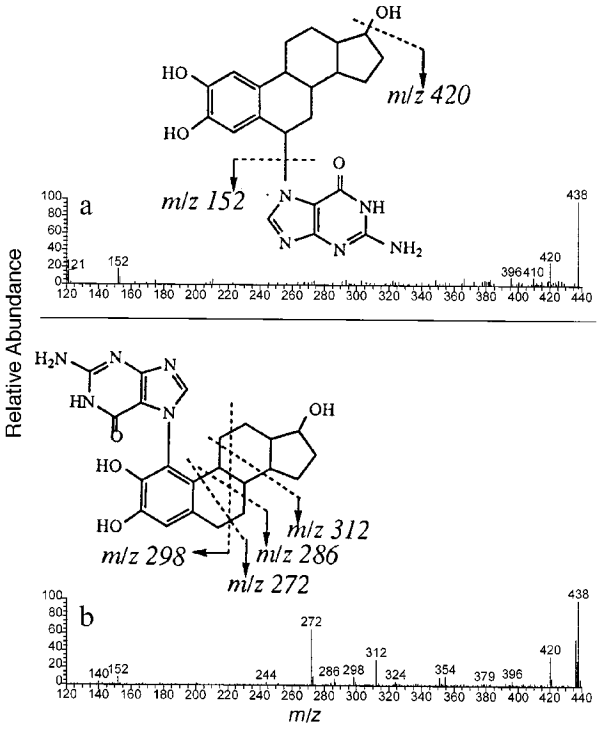

Fig. 2 CID mass spectra obtained from MH^+ ions (*m/z* 438) of, a, peak 2 and b, peak 6 from Fig. 1.

species. Thus, these two compounds may represent diastereoisomers, whose formation by nucleophilic attack of dG at the C6 prochiral atom of CE-quinones has already been raised.[2] On the other hand, the CID spectrum of the selected *m/z* 438 parent ion obtained from peak 6 displayed a completely different fragmentation pattern (Fig. 2b and Table 1). Indeed, in addition to the *m/z* 420 and the *m/z* 152 daughter ions which have been discussed above, the CID spectrum presented in Fig. 2b exhibited several diagnostic fragment ions at *m/z* 312, 298, 286 and 272, arising from charge-remote cross-ring cleavages occurring on the steroid skeleton, as previously described.[2] These fragment ions were characteristic of an addition of the base on the A ring of the steroid. Although it was not possible to determine whether the addition occurred at the C1 or the C4 position of the A ring of E-2,3-Q for peak 6, the use of LC-ESI-MS-MS allowed the discrimination of structurally different adducts as well as their rapid determination in a complex reaction mixture.

In the case of the M_r 553 adducts, two major compounds could be characterized by LC-ESI-MS analysis, respectively eluted at retention times (R_t) 29.5 min for peak 3 and 30.8 min for peak 4 (Fig. 1b). The full scan CID mass spectra of both the corresponding *m/z* 554 MH^+ ions resulted in superimposable product ion spectra, displaying *m/z* 438, *m/z* 287 and *m/z* 268 as the most abundant daughter ions (Table 1). The *m/z* 438 is generated by the loss of deoxyribose with proton transfer from the sugar to the guanine moiety, and the cleavage of the steroid-base bond leads to the *m/z* 287 (2-hydroxy-17β-estradiol carbocation) and *m/z* 268 (protonated deoxyguanosine) ions. In addition, a third minor M_r 553 adduct was detected (peak 5, R_t 35.6 min, Fig. 1b). The corresponding CID spectrum obtained from the *m/z* 554 parent ion exhibited the *m/z* 438 daughter ion but not the *m/z* 287 and 268 ions (Table 1). Thus, the LC-ESI-MS-MS analysis allowed us to discriminate two different kinds of the M_r 553 adducts.

The CID mass spectrum obtained from MS^3 on the *m/z* 438 ion isolated from the decomposition of the MH^+ ion (*m/z* 554) for peak 4 displayed the two complementary fragment ions at *m/z* 287 (2-hydroxy-17β-estradiol cation) and *m/z* 152 (protonated guanine), arising from the simple cleavage of the steroid from the base (Table 1). The MS^3 product ion spectrum of peak 3 was identical to that obtained from peak 4. Thus as for peaks 1 and 2, it may be that peaks 3 and 4 were diastereoisomeric adducts in which dG was linked to the C6 atom of the steroid *via* its N2 exocyclic nitrogen atom (since the nucleophilic attack of other nitrogen atoms of dG should have led to the destabilization of the sugar-base bond). On the other hand, the MS^3 product ion spectrum of *m/z* 438 ion selected from the decomposition of *m/z* 554 ion obtained from peak 5 using the same experimental excitation conditions showed a completely different fingerprint for this compound compared with peaks 3 and 4. Actually, this MS^3 spectrum is very close to the CID spectrum obtained from the *m/z* 438 MH^+ ion of peak 6 (Table 1). In particular, the fragment ions generated by charge-remote fragmentation processes were diagnostic ions of the linkage of the base on the aromatic ring of the steroid.[2,3] Thus, it could be concluded that peak 5 was an adduct in which dG was linked to the C1 or the C4 atom of the steroid *via* its N2 exocyclic nitrogen atom.

In conclusion, the detection of various M_r 437 adducts which were not previously mentioned[2,3] in the reaction of estrogen-2,3-quinones with dG showed that 2-hydroxy-estrogens do not form only stable (M_r 553) adducts, but are also capable of producing depurinating adducts. The ability to perform LC-ESI-MS^3 experiments proved to be very effective in the characterization of a minor M_r 553 adduct (peak 5), indicating that the C6 position of the steroid may not be the only electrophilic site involved in the formation of 2,3-CE adducts by Michael addition as previously reported.[2]

Table 1 Main diagnostic fragment ions observed in the MS^n product ion spectra of peaks 1 to 6 from Fig. 1.

Peak number	MS^n experiment	Fragment ions (*m/z*)
1	MS-MS 438→	420 - 269 - 152
2	MS-MS 438→	420 - 269 - 152
3	MS-MS 554→	438 - 287 - 268
	MS-MS-MS 554→ 438→	287 - 269 - 173 - 152
4	MS-MS 554→	438 - 287 - 268
	MS-MS-MS 554→ 438→	287 - 269 - 173 - 152
5	MS-MS 554→	438
	MS-MS-MS 554→ 438→	420 - 324 - 312 - 298 - 286 - 272 - 152
6	MS-MS 438→	420 - 324 - 312 - 298 - 286 - 272 - 152

Reaction between E-2,3-Q and dC

The reaction of E-2,3-Q with dC has been investigated in the same way as for dG, *i.e.* using the same reaction conditions as well as the same LC-MS-MS analysis procedure. Several unpolar and partially resolved compounds were detected at the end of the gradient (R_t 45–50 min). They corresponded to previously described covalently dimeric forms[31,32] of E-2,3-Q (not indicated by UV detection, Fig. 3a), evidenced by their respective MS-MS spectra (data not shown). Only one peak which could correspond to an expected adduct has been detected. This peak was eluted at R_t 25.5 min (Fig. 3b) and gave a peak at *m/z* 514, corresponding to the MH^+ ion for the addition of one molecule of dC to E-2,3-Q. This result constitutes the first evidence for the formation of a dC–CE adduct under non-reductive conditions.[2,5] Actually, no reaction product between CE quinones and dC could be observed in previous investigations[2] carried out under the same acidic conditions as were used in this work. Using reductive conditions in solution in order to generate a radical anion from the estrone-3,4-quinone, Akanni *et al.*[5] could observe the formation of two different adducts in which the exocyclic N4 atom of dC was linked to the C1 and C2 atoms of the steroid, respectively. However, the radical active species used in this work is particular and leads to the exclusive formation of adducts bound to the A ring of the steroid.[4,5,32] In

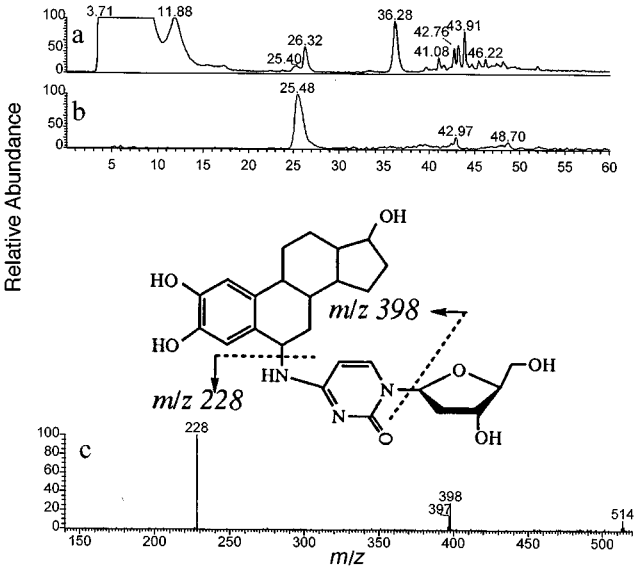

Fig. 3 LC-ESI-MS-MS analysis of a E-2,3-Q–dC crude reaction mixture. a, UV chromatogram (λ = 280 nm), b, reconstructed selected ion chromatogram for *m/z* 514 and c, CID mass spectrum obtained from the MH^+ ion (*m/z* 514) of the 2-hydroxy-17β-estradiol–dC adduct (R_t 25.48 min.).

our case, using full-scan MS-MS analysis on the *m/z* 514 ion, the product ion spectrum reported in Fig. 3c was obtained. It displayed daughter ions at *m/z* 398 ([MH-deoxyribose]$^+$) and *m/z* 228 (protonated dC), respectively, meaning that under collisional activation conditions into the ion trap, the *m/z* 514 parent ion decomposed according to simple cleavages of the sugar-base and base-steroid bonds, respectively, as we reported in a previous work.[28] This fragmentation pathway is common to several other CE adducts in which the C6 position of the steroid is involved,[2] as for peaks 3 and 4 from dG (Table 1). Work is now in progress in order to achieve the complete structural identification of this compound. Nevertheless, this constituted the first observation of an adduct of dC with CE in acidic medium.[2,5] Compared to dG, it can be concluded that under the same reaction conditions, dC yielded only a stable adduct since no adduct involving the loss of deoxyribose has been detected.

Conclusion

Data have been presented concerning the characterization of several adducts by direct injection of the crude reaction mixtures onto an LC-ESI-MS-MS system. In addition, the use of multisequential MS 'to the third' experiments proved to be very useful for discriminating adducts where MS-MS experiments could not. To our knowledge, this represents a first application of LC-ESI-MS-MS to the study of reactions between CE quinones and deoxyribonucleosides, except the very recent work by Bolton *et al.* on equinelin.[23]

In conclusion, although the multisequential MS^n techniques used in this work did not allow a precise structural identification for each adduct formed in the various reactions, LC-ESI-MS^n should be considered as a very powerful analytical tool for the detection of suspected DNA adducts as estrogen bound metabolites generated *in vivo*. More generally, the usefulness of LC-ESI-MS^n technique should not be limited to the detection of estrogen DNA adducts but should also be extended to the tracking of putative DNA adducts formed from other drugs or xenobiotic molecules which could undergo a preliminary oxidation step, thus generating potential DNA alkylating agents.

Acknowledgements

The authors gratefully acknowledge the Institut Scientifique Roussel for financial support to this work (C. Van Aerden's forthcoming thesis). We thank Dr. E. Rathahao for her help in LC-MS analyses.

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Paper 8/05126G