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Interpretation of Electrospray and Atmospheric Pressure Chemical Ionization Mass Spectra of 10-Formyl-7,8-dihydrofolic Acid and 5-Formyl-5,6,7,8-tetrahydropteroic Acid

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Interpretation of positive- and negative-ion electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectra of 10-formyl-7,8-dihydrofolic acid (10-FDHFA) and 5-formyl-5,6,7,8-tetrahydropteroic acid (5-FTHPA) is discussed. ESI mass spectra enable unambiguous molecular weight (MW) determination. In addition to the determination of MW, APCI mass spectra also make possible structure elucidation of 10-FDHFA and 5-FTHPA. ESI and APCI are complementary ionization techniques and appear to be useful alternatives to conventional electron ionization (EI) for the structure elucidation of non-volatile carboxylic acids. Prior to mass spectral analysis, the acids investigated were separated by reverse-phase high-performance liquid chromatography (HPLC). Copyright © 1999 John Wiley & Sons, Ltd.

ESI and APCI are the most often used ionization techniques in the contemporary practice of coupled high-performance liquid chromatography/mass spectrometry (HPLC/MS). Conventional EI is the preferred ionization technique for structural elucidation,[1] but it cannot be applied for non-volatile or thermally labile compounds, such as compounds containing more than one carboxylic and especially sulphonic acid groups. Monosulphonic aromatic acids with none or one other functional group (CH_3, OH or Cl) are the most polar compounds, whose EI mass spectra have been reported using the HPLC/MS technique with a particle beam interface.[2]

Folate compounds are found in various cereal-grain products,[3] and 5-formyl-5,6,7,8-tetrahydrofolic acid is important in cancer chemotherapy.[4] 10-FDHFA was found in the cytosol of methotrexate-treated MCF-7 breast cancer cells.[5] A densitometric thin-layer chromatographic method for the determination of folate impurities in leucovorin calcium has been described.[6]

In the present work, ESI and APCI mass spectra of 10-FDHFA and 5-FTHPA are investigated. These spectra can be used not only for the MW determination, but may provide valuable additional information useful for the structural elucidation of folate compounds. The correlation between the suggested structures and observed fragment ions was studied and the composition of fragment ions was suggested. To our knowledge, mass spectra of these acids and of other folate compounds have not been published so far.

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EXPERIMENTAL

Materials

Methanol for HPLC was received from Baker (Deventer, The Netherlands), water was double-distilled in glass with addition of potassium permanganate. Ammonium acetate was purchased from Sigma-Aldrich (Prague, Czech Republic). Samples were obtained as solutions in 5% methanol/95% 5 mM ammonium acetate in water from a pharmaceutical company. The purity of 10-FDHFA (85%) and 5-FTHPA (99%) was determined from the HPLC chromatograms using the normalized peak areas method.

High-performance liquid chromatography

The chromatographic apparatus consisted of a Waters 616 pump, a Waters 996 diode-array detector and a Waters 717+ autosampler (all from Waters, Milford, MA, USA). The octadecyl silica glass cartridge column, Separon SGX C18 (150 × 3 mm i.d., 7 μm particle size) was purchased from Tessek Ltd. (Prague, Czech Republic). The mobile phase was filtered through a 0.45 μm Millipore filter prior to use and degassed by continuous stripping by a stream of helium. The pre-mixed mobile phase consisted of 5% methanol and 95% 5 mM ammonium acetate in water. 10 μL sample volumes were injected and the flow rate of the mobile phase was kept at 1 mL/min in each experiment. The retention times of 10-FDHFA and 5-FTHPA were 2.3 and 4.5 min, respectively.

Mass spectrometry

The effluent from the liquid chromatograph was directly introduced into a quadrupole mass spectrometer VG Platform (Micromass, Manchester, UK) equipped with ESI and APCI probes operated in positive- or negative-ion mode.

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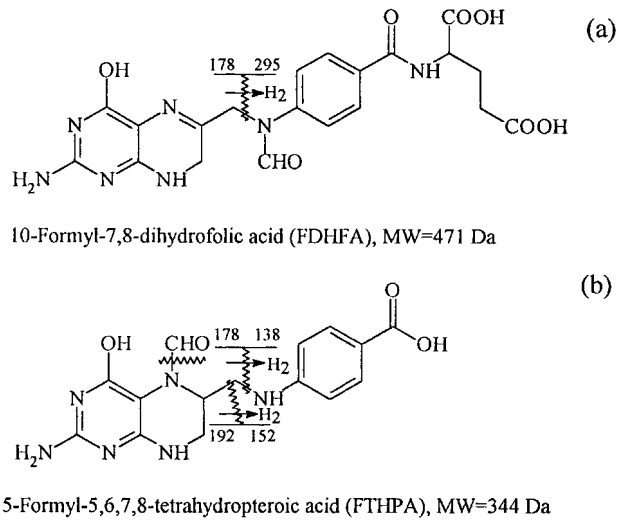

Figure 1. Structures of (a) 10-formyl-7,8-dihydrofolic acid (10-FDHFA) and (b) 5-formyl-5,6,7,8-tetrahydropteroic acid (5-FTHPA).

The data was acquired in the *m/z* range 35–600 at 1.9 s per scan. In the APCI mode, the temperature of the ion source and of the probe were 100 and 500 °C, respectively. The temperature of the ESI ion source was kept at 100 °C. The effluent split ratio 1:20 was used before the introduction of the eluate into the ESI probe of the mass spectrometer. The cone voltage was set at 10 V for positive-ion APCI, at 20 V for negative-ion APCI and at 30 V for negative- and positive-ion ESI.

RESULTS AND DISCUSSION

The structures of the two compounds studied are shown in Fig. 1 with an attempted explanation of the main fragmentation paths.

10-Formyl-7,8-dihydrofolic acid (10-FDHFA)

From the negative-ion ESI mass spectrum of 10-FDHFA, a MW of 471 Da could be easily determined as only the following ions were found in the mass spectrum: $[M - H]^-$ (*m/z* 470, relative abundance 100%), $[M - 2H + Na]^-$ (*m/z* 492, 6%), $[M - 2H + K]^-$ (*m/z* 508, 2%) and the fragment ion with *m/z* 293 (14%). The positive-ion ESI mass spectrum of dicarboxylic acid 10-FDHFA cannot be measured in contrast to monocarboxylic acid 5-FTHPA.

The differences between the positive- and negative-ion APCI mass spectra of both studied compounds correspond to two mass units, hence only positive-ion mass spectra are presented. The characteristic ions $[M + H]^+$ (*m/z* 472, 8%) and $[M + Na]^+$ (*m/z* 494, 1%) in the positive-ion APCI mass spectra (see Fig. 2) and $[M - H]^-$ (*m/z* 470, 2%) and $[M - 2H + Na]^-$ (*m/z* 492, 0.5%) in the negative-ion mode spectra allow unambiguous confirmation of the MW of 10-FDHFA. In spite of the low intensities of the corresponding peaks, the mass difference Δ *m/z* = 22 is characteristic and cannot be misinterpreted.

In addition to the MW determination, structural information can be obtained from the abundant fragment ions in APCI mass spectra. The suggested structures of fragment ions (Fig. 2) are in good agreement with the structure of 10-FDHFA. For better clarity, Fig. 3 shows the magnified low-abundant fragment ion region from m/z 182 to 517 with suggested structures of the important fragment ions.

Two most abundant fragment ions are observed due to the cleavage of the bond between C_9 and N_{10} (see Fig. 1(a)). When the charge is retained on the carbon C_9, the ion at *m/z* 178 (100%) is formed. The relative abundances of all other ions are lower than 20%, which can be explained by the resonance stabilization of the ion with *m/z* 178. The ion at m/z 180 (26%) probably corresponds to the ion *m/z* 178 with one saturated double bond. The ion *m/z* 161 (3%) is formed by the loss of a neutral molecule of ammonia from *m/z* 178.

The ion at *m/z* 295 (18%) corresponds to the immonium ion formed by the cleavage of the bond between C_9 and N_{10}.

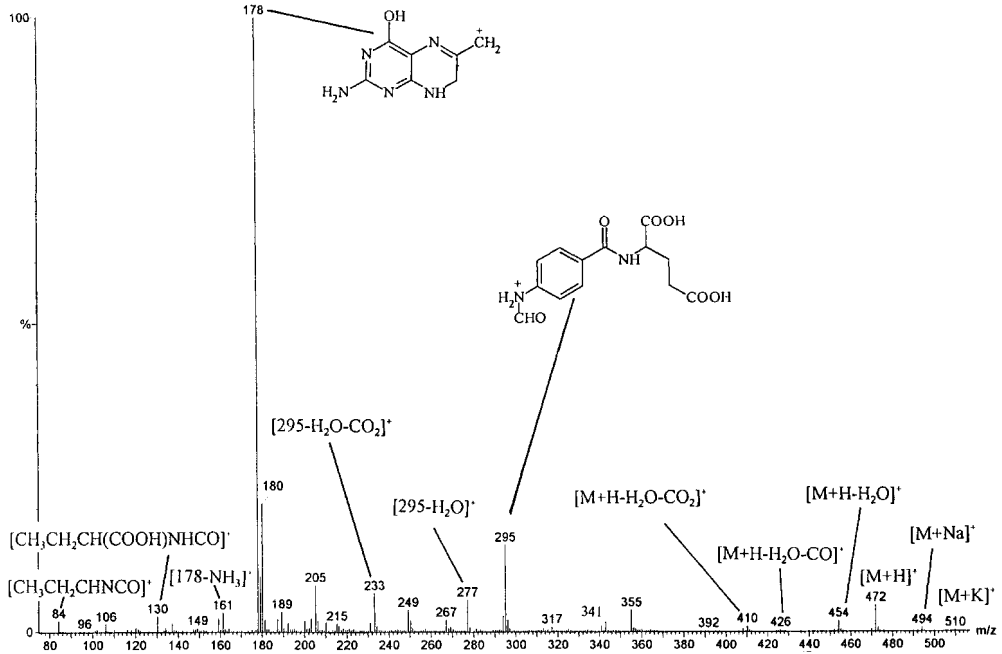

Figure 2. Positive-ion APCI mass spectrum of 10-formyl-7,8-dihydrofolic acid.

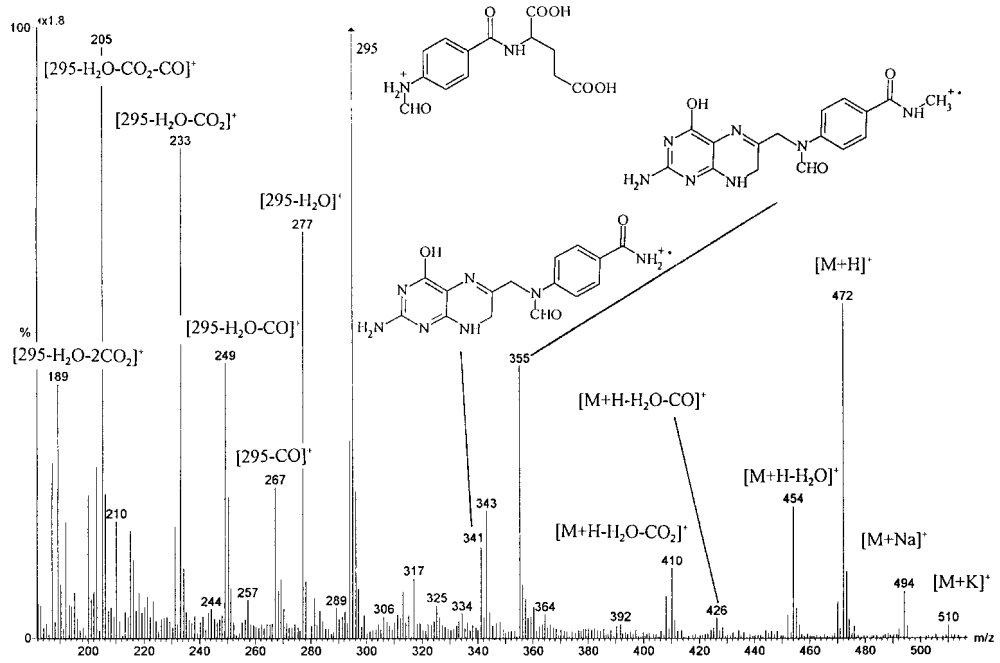

Figure 3. Positive-ion APCI mass spectrum of 10-formyl-7,8-dihydrofolic acid (a magnified region from *m/z* 182 to 517).

Other fragment ions correspond to the subsequent losses of neutral molecules of water, carbon monoxide and dioxide from the ion at *m/z* 295, such as $[295 - H_2O]^+$ (*m/z* 277, 6%), $[295 - CO]^+$ (*m/z* 267, 2%), $[295 - H_2O - CO]^+$ (*m/z* 249, 4%), $[295 - H_2O - CO_2]^+$ (*m/z* 233, 7%), $[295 - H_2O - CO_2 - CO]^+$ (*m/z* 205, 9%) and $[295 - H_2O - 2.CO_2]^+$ (*m/z* 189, 3%). Similar losses of neutral molecules are observed from the protonated molecule: $[M + H - H_2O]^+$ (*m/z* 454, 3%), $[M + H - H_2O - CO]^+$ (*m/z* 426, 0.5%) and $[M + H - H_2O - CO_2]^+$ (*m/z* 410, 1%). The ion at *m/z* 130 (3%) probably belongs to the $[CH_3CH_2CH(COOH)NHCO]^+$ ion. Further loss of formic acid yields the ion at *m/z* 84 (1%).

All the observed ions have an even number of electrons, which is typical for APCI mass spectra. Odd-electron ions are rarely observed and, if present, their intensities are usually low. The low abundant ions with *m/z* 341 (1%) and *m/z* 355 (5%) are probably odd-electron ions (see Fig. 3).

5-Formyl-5,6,7,8-tetrahydropteroic acid (5-FTHPA)

A MW of 344 was determined from the masses of the ions

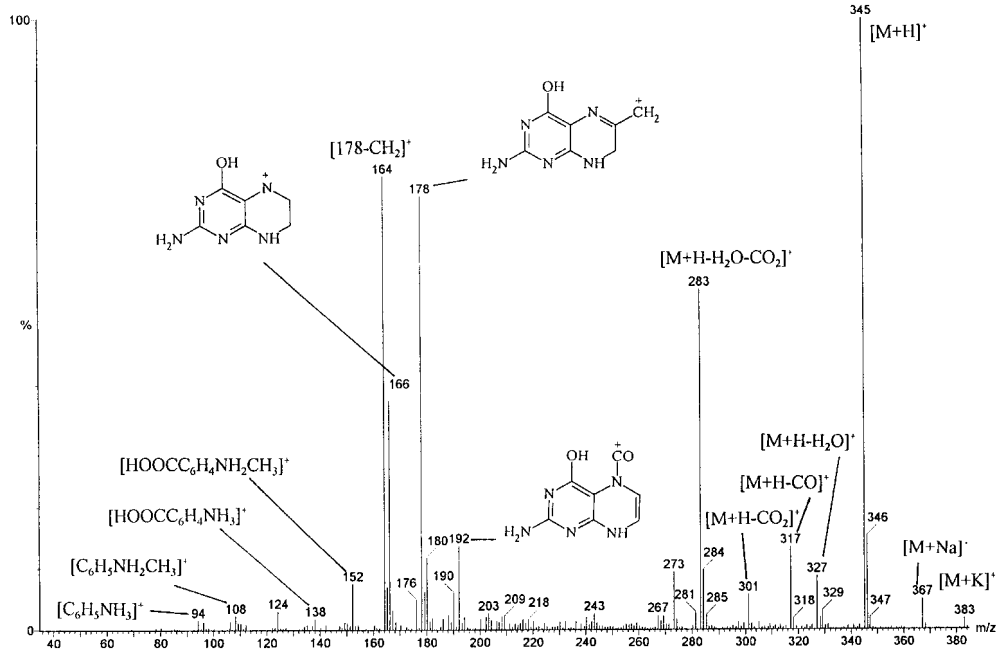

Figure 4. Positive-ion APCI mass spectrum of 5-formyl-5,6,7,8-tetrahydropteroic acid.

$[M + H]^+$ (*m/z* 345, 100%), $[M + Na]^+$ (*m/z* 367, 9%) and $[M + K]^+$ (*m/z* 383, 1%) in the positive-ion ESI mass spectra and of the ions $[M - H]^-$ (*m/z* 343, 100%), $[M - 2H + Na]^-$ (*m/z* 365, 9%) and $[M - 2H + K]^-$ (*m/z* 381, 2%) in the negative-ion ESI mass spectra. The identical ions are also observed in the APCI mass spectra. The relative abundances of these ions in the APCI mass spectra are higher than the analogous ions of 10-FDHFA, which can possibly be explained by the easier fragmentation of dicarboxylic acid 10-FDHFA.

Similar to 10-FDHFA, the positive-ion APCI mass spectrum of 5-FTHPA (Fig. 4) shows ions formed by characteristic subsequent losses of H_2O, CO and CO_2: $[M + H - H_2O]^+$ (*m/z* 327, 9%), $[M + H - CO]^+$ (*m/z* 317, 13%), $[M + H - CO_2]^+$ (*m/z* 301, 6%), $[M + H - H_2O - CO_2]^+$ (*m/z* 283, 56%) and $[M + H - CO_2 - CO]^+$ (*m/z* 273, 9%). The cleavage of the bond between C_6 and C_9 may give rise to two ions: the first ion with the charge retained on C_9 $-[HOOCC_6H_4NH_2CH_3]^+$ (*m/z* 152, 7%) or the second ion with the charge retained on C_6 (*m/z* 192, 14%). The peaks observed in the low-mass region can be attributed to the series of protonated amine ions: $[HOOCC_6H_4NH_3]^+$ (*m/z* 138, 2%), $[C_6H_5NH_2CH_3]^+$ (*m/z* 108, 2%) and $[C_6H_5NH_3]^+$ (*m/z* 94, 2%). The ion with *m/z* 178 (71%) probably has the same structure as in this peak in the spectrum of 10-FDHFA, but a lower relative abundance, because its formation requires consecutive cleavages of two bonds, whereas one bond cleavage is sufficient for 10-FDHFA. The ion with *m/z* 164 (74%) corresponds to the loss of carbon monoxide from the ion with *m/z* 192 or a CH_2 group from the ion with *m/z* 178. The less intensive peaks at *m/z* 166 (37%) and 180 (12%) correspond to analogous ions with *m/z* 164 and 178 with one saturated double bond. The negative-ion APCI mass spectrum of 5-FTHPA is very similar to the positive-ion APCI mass spectrum with *m/z* values lower by two units, therefore it is not discussed.

CONCLUSIONS

This work presents an example of the use of a single stage quadrupole mass spectrometer with electrospray and atmospheric pressure chemical ionization not only for molecular weight determination, but also for the verification of suggested structures of strongly polar non-volatile compounds. We believe that the fragmentation patterns in the APCI mass spectra of 10-formyl-7,8-dihydrofolic acid and 5-formyl-5,6,7,8-tetrahydropteroic acid suggested in this work can be useful for the identification of other derivatives of folic and pteroic acids. High resolution mass spectrometry, tandem MS/MS or NMR techniques can provide for unambiguous confirmation of suggested structures, but the detailed interpretation of APCI mass spectra may provide useful structural information even with a simple bench-top instrument.

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