Quantitative Field Desorption Mass Spectrometry
XIX. Determination of Tritium in Steroids*
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Summary. The first application of field desorption mass spectrometry (FD-MS) to the determination of the label content and the statistical isotopic distribution in radiolabelled substances is reported. The total amount of tritium in steroids such as [6,7-3H]estradiol and [2,4,6,7-3H]estradiol was estimated. Data are easily derived from the field desorption mass spectra which allow the calculation of the distribution of the radioisotope in the investigated biochemicals with an accuracy below 1%.

The two main advantages of the technique reported here are the small sample consumption in the nanogram range (which is particularly important for radiolabelled chemicals of high specific activity) and the high-molecular ion abundance of polar substances (the thermal and mass spectrometric fragmentation is almost completely missing) which give optimal information about the isotopic composition of the intact molecule. In addition, the short analysis time by FD-MS of approximately 30 min for the complete isotopic determination and the good precision of a few tenths of a percent underline the utility of this new method for the assay of radioisotopes in compounds of biochemical, medical and environmental interest.

Key words: Best. von Tritium in Steroiden; Massenspektrometrie; Felddesorption; Isotopenverteilg. in Biochemikalien

Introduction

The use of radiolabelled compounds [11, 13, 18, 28] in biochemical and medical research has found wide application. Due to the sensitivity of detection, trace quantities of substances can be measured. The isotopes most frequently used in labelling experiments are carbon-14, tritium, sulphur-35, phosphorus-32, and iodine-125. Therefore a substantial number of radioactive substances are commercially available tagged with these isotopes.
Tritium labelled substances present interesting analytical problems. The analysis of these materials is made very difficult because of the small quantities prepared. The ever increasing need for higher specific activities (i.e. radioactivity/weight) requires the development and application of new sensitive tools for analysis.

As the specific activity rises, for a given number of counts, the weight is smaller (e.g. [6,7-3H]estradiol, specific activity 60 Ci/m mole, 100 µCi = 0.46 µg; [2,4,6,7-3H]estradiol, specific activity 115 Ci/m mole, 100 µCi = 0.23 µg; [2,4,6,7,16,17-3H]estradiol, specific activity 170 Ci/m mole, 100 µCi = 0.17 µg).1

Currently used analytical techniques are designed to determine identity, purity, specific activity (the latter being accomplished by computation from separate measurements of weight and counts per minute) and labelling position. These include then, chromatographic mobility [13] (identity and radiochemical purity), spectroscopic measurements (identity, weight and chemical purity), liquid scintillation counting [12] (counts per minute), Nuclear Magnetic Resonance (NMR) absorption [3] (labelling position).

This report presents preliminary results on the application of field desorption mass spectrometry (EI-MS) for the analysis of carbon-14 labelled compounds has been reported [7, 9]. We felt that FD-MS, a method that gave no or minimum fragmentation for a wide variety of biochemicals and natural products [22], would supply us with information not well obtained by the other methods.

It would, thus, easily identify the compound, give a qualitative picture of purity (both chemical and radiochemical), indicate the amounts of the different labelled species of the compound present and the specific activity.

Previous work by FD-MS showed that using a multichannel analyzer and integrating electrical recording of a large number of cyclic magnetic scans the natural abundance of isotopes in drugs, pesticides and natural products can be determined with an average error of 0.4% [16]. For sample amounts in the micromole range the deuterium content and distribution was estimated in sugars, biogenic amines and anticancer drugs. The first quantitative results for the estimation of 13C in [1,13C]glucose were obtained by attachment of monoisotopic cations and gave a mean error of the individual measurements of about 7%. This value decreased on evaluation of 100 scans to ±0.7% [15]. The studies for the estimation of the label content of stable isotopes were an essential prerequisite for the development and use of quantitative FD-MS. Endogenous compounds such as dopamine and some of its metabolites [14], and choline and acetylcholine [17] were determined in physiological fluids and tissues. Further, a novel technique for determining the hydride transfer stereospecificity of nicotinamide adenine dinucleotide linked oxidoreductases has been described [8]. In this case FD and EI-MS were used in combination for the assay of 3H incorporated after enzymatic reaction.

Ultratrace analysis of metals using stable isotopes of thallium [25] in biological samples such as brain tissue were reported for nanogram amounts without pretreatment. These investigations showed that FD-MS in conjunction with the isotopic dilution method possesses good sensitivity, precision and accuracy for routine quantitative analysis and can be regarded as promising method for biochemical and medical analysis [21, 23, 26].

To our knowledge, this is the first report of the application of FD-MS to tritium labelled substances. The question was whether the FD technique allows the determination of the content and distribution of the label in a radiolabelled, underivatized and non-volatile substance under realistic analytical conditions (sample amount, purity etc.).

Experimental

The mass spectrometric studies were performed on a double focussing mass spectrometer of type Varian MAT 731 with a combined EI/FI/FD ion source. The emitters used were 10 µm diameter tungsten wires activated at high temperature with an average length of the microneedles of 50 µm. The samples were applied by the modified syringe technique and desorbed by direct heating (emitter heating current) or indirect heating utilizing the radiation of a tunable argon ion laser (Spectra Physics model 166, 514 nm line) [27].

The FD ion currents were recorded electrically and processed by a Varian MAT SS100 data system or accumulated by a multichannel analyzer of type CAT-3024 Varian which was triggered from the cyclic magnetic scan of the mass spectrometer [16, 24]. The model compounds, unlabelled estradiol, [6,7-3H] and [2,4,6,7-3H]estradiol were supplied by New England Nuclear, Boston, USA.

Results and Discussion

Figure 1a shows the averaged FD mass spectrum of estradiol with a natural abundance of isotopes which was recorded by conventional electric recording and acquisition of the ion signals by a data system. The well established and characteristic feature of FD-MS to give
abundant molecular ions and only minor fragmentation is again clearly demonstrated for the steroid hormone. It is noteworthy that the standard compound (high purity quality) gives no ions that could be attributed to small accompanying impurities. Since only 500 ng were consumed for the complete measurement, the FD technique appears to be highly sensitive and thus useful for investigations of tritium labelled compounds of high activity.

Figure 1b gives a first example of an FD analysis of a radiolabelled substance. When a mixture of cold and [6,7-3H]labelled estradiol was recorded under the same conditions as described for the unlabelled standard two facts are clearly discerned. First, the content of tritium label is at least qualitatively indicated for one $^3$H at $m/z$ 274 by the slight increase in ion abundance, the majority of the [6,7-3H]estradiol molecules contributes to the abundant molecular ion at $m/z$ 276 and finally some higher content of three and four tritium atoms can be assumed in view of the abundances of the ions at $m/z$ 278 and $m/z$ 280. Second, a plurality of weak ion signals are recorded and give a hint that some impurities are present although these compounds must be of very minor concentration.

In order to perform a more reproducible and reliable direct isotope determination of unlabelled and radiolabelled estradiol a smaller mass range including the molecular ions was recorded by a multichannel analyzer using repetitive magnetic scans. A detailed description of this technique for direct isotope determination by FD-MS has recently been given [16, 19, 26] and its basic advantage is to compensate for weak and fluctuating ion currents if a sufficient number of scans can be accumulated. The results are shown in Fig. 2 for unlabelled estradiol: $m/z$ 272 100% rel. abundance (theor. 100%); $m/z$ 273 20.5% rel. abundance (theor. 20.6%); $m/z$ 274 2.7% rel. abundance (theor. 2.5%); $m/z$ 275 0.3% rel. abundance (theor. 0.2%).

One difficulty in the determination of isotopes by FD-MS should be mentioned. Field-induced processes on the surface of the FD emitter such as proton transfer reactions may obscure the measurements of isotopic distributions. In particular at higher emitter temperatures the formation of $[M + H]^+$, $[M - H]^+$ and $[M - 2H]^+$ ions occurs and simultaneously the accuracy in isotope determination decreases. In Fig. 3 the onset of the formation of $[M + H]^+$ and $[M - 2H]^+$ ions of estradiol (cold, standard purity) is illustrated. As may be inferred from the data in this figure approximately 1% relative abundance of the $[M - 2H]^+$ ion is a distinct sign that owing to disproportional reactions on the emitter surface the coincident formation of $[M + H]^+$ ions is responsible for the finding that $m/z$ 273 is about 0.6% too high. Thus it becomes clear...
that for direct isotope determination by FD-MS the temperature of the emitter and sample have to be kept at the lowest possible value which just allows the registration of ion currents of sufficient intensities. Alternatively, cationization by mono-isotopic metal cations [15] or conversion of the sample molecule into an organic cation [10, 20] can be performed and thus high sensitivity and good accuracy are achieved.

For direct isotope determination of the same mixture of unlabelled and radiolabelled estradiol as described in Fig. 1 b the molecular ion region from $m/z$ 267 to $m/z$ 285 was scanned repetitively and the obtained ion currents were accumulated with the multichannel analyzer. The FD spectrum recorded under these conditions (Fig. 4) gives information about the total radiolabel content and its distribution. According to the ratio of the weights of unlabelled and radiolabelled substance the evaluation of the peak heights revealed that 2% $[\text{H}]_3$estradiol, 11.9% $[\text{H}]_4$estradiol, 69.8% $[\text{H}]_2$estradiol are present in the labelled biochemical. In contrast to the information indicated in the single scan FD spectrum in Fig. 1 b a significant contribution of estradiol containing four tritium atoms was not detected here. From these results if can be derived that the total label content is 8.4 %, this means that 8.4% of the hydrogen in estradiol is exchanged against tritium.

Figure 5 shows the FD spectrum of $[2,4,6,7-^{3}\text{H}]$estradiol. At the beginning of the FD experiments (at 0 mA emitter heating current) an impurity at $m/z$ 279 was found. Although the intensity of this ion

**Fig. 2**
FD mass spectrum of estradiol with a natural abundance of isotopes. The mass range between $m/z$ 268 and $m/z$ 278 was recorded electrically using 60 repetitive scans and accumulating the FD signals with the multichannel analyzer. A detailed description of this procedure has been given in ref. [16]. The sample consumption was 500 ng from a solution of benzene/EtOH (9:1), the emitter heating current was raised from 0 -- 10 mA.

**Fig. 3**
Direct isotope determination of the unlabelled estradiol standard using the experimental conditions as described in Fig. 2. In this case, however, indirect heating by laser radiation was performed in order to reduce thermal stress as far as possible [27]. The onset of field-induced reactions is clearly indicated by the $[\text{M} - 2\text{H}]^{+}$ ion
decreased rapidly with increasing emitter temperature a small contribution of its isotopic peak at m/z 280 has to be considered for the calculation of the label content and distribution in labelled estradiol. From our previous analytical experience and due to the relative high volatility it is assumed that a plasticiser such as octylhydrogenphthalate or dibutylphthalate was present in the sample. The results of the tritium determination gave 1% [3H0], 4.4% [3H1], 18.5% [3H2], 41.9% [3H3] and 32.3% [3H4] and 2% [3H5] estradiol and correspondingly the tritium label content is 12.7%.

The precision of the isotopic assay by FD is a few tenths of a percent. The pilot studies described above did not define the accuracy of the method. However, strong evidence from other isotopic determinations, for instance of metals, which were confirmed by atomic absorption spectroscopy [1], of drugs labelled with stable isotopes (confirmed by field ionization and electron impact mass spectrometry [4]) and of vitamin B12 derivatives controlled by conventional combustion analysis [20] and recent parallel investigations of 14C labelled glucose by NMR and FD [2], suggested that an accuracy below 1% on an absolute basis can be expected.

**Conclusion**

Above all, isotopic determinations by FD of stable and radiolabelled chemicals can be utilized for quality control of non-volatile and/or thermally labile substances. Further, the exact knowledge of the label content is an essential prerequisite for quantitative FD-MS using the isotope dilution technique [23]. In addition, it has been demonstrated that the isotopic pattern can be useful for reliable interpretation of high resolution data [10, 20] and for the detection of biological enrichment or depletion processes. In general, the advantages of the method reported are sensitivity, specificity and reliability of the data for the label content and, in particular, the small amount of sample and of radioactivity handled.

However, in tritiated steroids for use as tracers in medical or biochemical research, it is important to determine not only the position but also the stereochemistry. In this respect 3H NMR appears to be a well suited complementary tool although much more instrument time and sample is required. Indeed, a combination of both techniques, FD-MS and 3H NMR should offer an optimum for quality control of tritium labelled steroids.
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References

12. Kobayashi, Y., Maudsley, D. V.: In: Biological applications of liquid scintillation counting. XXX

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