DIRECT ISOTOPE DETERMINATION OF DRUGS LABELED WITH STABLE ISOTOPES OF HYDROGEN, CARBON, AND NITROGEN BY FIELD DESORPTION MASS SPECTROMETRY[†]

U. Bahr and H.-R. Schulten[Δ]
Institute of Physical Chemistry
University of Bonn
Wegelerstr. 12, 5300 Bonn 1
Federal Republic of Germany

SUMMARY

By recording the molecular ion group with repetitive magnetic scanning and accumulation of the mass spectra in a multichannel analyser field desorption mass spectrometry was used to determine the isotopic abundances of three widely used drugs in their unlabeled and stable-isotope labeled forms. The label content and statistical distribution of ^{2}H, ^{13}C and ^{15}N in analogs of an antitumor agent, cyclophosphamide and one of its metabolites 4-ketocyclophosphamide and of two analgesics antipyrine and carbamazepine were investigated. Using nanogram amounts of sample the precision and accuracy of the measurements achieved were below ±1% on an absolute basis.

Key Words: Stable Isotopes, Quantitation, Mass Spectrometry, Field Desorption, Antineoplastic and Analgesic Agents

INTRODUCTION

In several areas of drug research, drugs and metabolites are utilized in which one or more atoms have been replaced by stable isotopes [1]. The most common isotopes used are ^{2}H, ^{13}C, ^{15}N and

[†]Quantitative Field Desorption Mass Spectrometry: Part XVII, for part XVIII see reference 14.
[Δ]To whom correspondence should be addressed.

0362-4803/81/040571-11$01.00

Received November 9, 1979
Revised December 20, 1979

^{18}O and compounds labeled by these stable isotopes increasingly replace radioactively labeled drugs by in vivo investigations, particularly in man.

For quantitative determinations by mass spectrometry (MS) the use of stable isotope dilution analysis is the method of choice. However, the accuracy of the results depends decisively on the accuracy of the isotope determination and the information about the label content and distribution of the compound. Among the various mass spectrometric ionisation methods, field desorption (FD) [2,3] has an exceptional position, as even compounds of high thermal lability and low volatility give molecular ions of high relative abundances. Therefore, the method is well suited for determination of the label content of a compound [4]. Using, for instance, electron impact (EI) ionisation the estimation of the label content of those compounds often has to be performed on the isotopic abundances of fragment ion groups. Thus errors may be introduced due to scrambling and/or skeletal rearrangements. In contrast, in FD-MS ion currents are generated which consists almost exclusively of molecular or quasimolecular ions and hence this method is optimally suited for estimating the label content. A precision of $\pm$ 0.5% on an absolute basis can be achieved for the relative intensities of an ion group by taking an average over 30-100 repetitive magnetic scans. This averaging is best performed by accumulation of the signals via a multichannel analyser [4]. According to the chemical nature and purity of the sample between 100 pg and 10 ng are needed for one determination. The time requirement for one isotopic analysis is about 10 minutes.

In the following it will be demonstrated that using FD-MS for determination of isotopic abundances and the label content of a compound values of high precision and good accuracy can be obtained in a simple, rapid and reliable manner.

RESULTS AND DISCUSSION

The accurate ^{13}C-content of two ^{13}C labeled analogs of the analgesic drug antipyrine^{R}, (1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one, DAP), had to be determined as these analogs were used in breath tests of children [5]. The ^{13}C label was tagged in both N-methyl-groups. First, for examination of the accuracy of the method the FD, field ionisation (FI) and EI spectra of the molecular ion group of the unlabeled compound were recorded. The FD spectrum is shown in Figure 1a. The isotopic pattern was compared with the theoretical values for the natural isotopic abundances [6]. The theoretical values were calculated and plotted from a computer program after feeding the elemental composition of the compound into the system using the listed natural isotopic abundances. The comparison between the theoretical and found values in the FD mode gave a difference of 0.2%. From the good agreement of these values it can be assumed that under the applied experimental conditions neither H-elimination nor protonation of the molecule takes place. In this case the determination of the label content of ^{13}C DAP is considerably simplified and it can be directly calculated from the peak heights in the mass spectrometric pattern of the molecular ion of the ^{13}C labeled compound (Figure 1b). The signal at m/z 231 is due to the molecular ion of the unlabeled compound, the amount of it in the sample can be calculated from its peak height. At m/z 232 the molecular peak of the compound with one ^{13}C atom and at m/z 233 the molecular peak with two ^{13}C atoms are recognized. Indeed the heights of these two peaks contain a certain amount of the isotopic peaks from the unlabeled and singly labeled molecules of the compound. After correction of the peak heights the label content could be determined. In Table I the results of the isotopic determination by EI, FI and FD are shown.

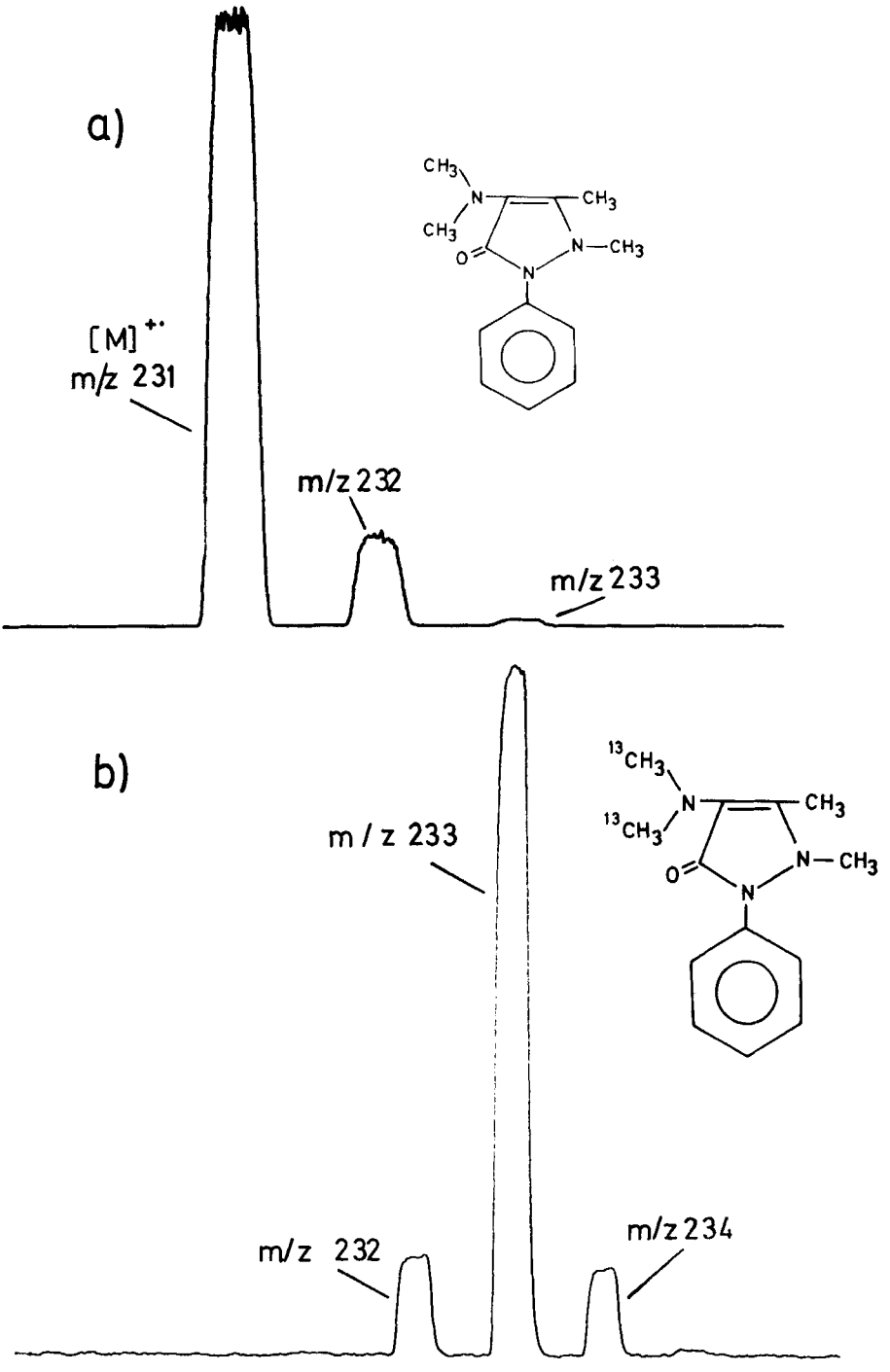

Fig 1. The FD mass spectra of the molecular group of the unlabeled antipyrine (a) and the sample 1 of the ^{13}C labeled analog (b) are shown.

Table I. Determination of the ^{13}C label content from two samples of ^{13}C enriched DAP. Comparison of the values obtained by FD, FI and EI mass spectrometry.

Formula	$C_{13}H_{17}N_3O$	$C_{12}{}^{13}C_1H_{17}N_3O$	$C_{11}{}^{13}C_2H_{17}N_3O$	$C_{10}{}^{13}C_3H_{17}N_3O$
Sample 1:				
FD-MS	9.6%	26.3%	56.4%	7.7%
FI-MS	9.5%	25.8%	57.5%	7.3%
EI-MS	10.1%	26.2%	56.3%	7.5%
Sample 2:				
FD-MS	0.6%	11.8%	77.4%	10.3%
FI-MS	0.6%	11.8%	77.9%	9.8%
EI-MS	0.9%	11.8%	78.3%	9.0%

In order to evaluate the capacity of MS for isotope determination it was of interest to investigate a compound by FD, FI and EI which gives abundant molecular ions with all techniques. The aim here was to obtain an estimate for the reproducibility, precision and accuracy for one model compound. Comparing the use of these different modes of ionisation for direct isotope determination of unlabeled and ^{13}C-labeled DAP the following facts emerged.

1. Field Desorption : For this relatively stable compound FD-MS yields only molecular ions and no significant field-induced processes are observed. Thus the estimation of the label distribution and ^{13}C content is facilitated. From the data in Table I it can be derived that sample 1 contains 12.5% ^{13}C and sample 2 15.2% ^{13}C calculated on the basis of the total number of carbon atoms in DAP.

2. Field Ionisation : The first determination of the ^{13}C content by FI-MS revealed similar results as FD. Again sample 1 gave 12.5% ^{13}C and sample 2 15.2% ^{13}C. However, when the abundances of unlabeled

DAP were estimated and compared with the theoretical values, a minor systematic error of approximately -0.9% for the [M + 1] ion occured which in part might be due to isotopic effects during evaporation of the sample from the direct introduction system.

3. Electron Impact : The ^{13}C content in sample 1 was found to be 12.4% and 15.1% for sample 2 and confirmed the FI/FD results. The standard deviations for all employed techniques were between 0.01 and 0.1%.

In the same manner the label content of carbamazepine (5 H-dibenz [b,f] azepine-5-carboxamide),which was labeled with ^{15}N in the

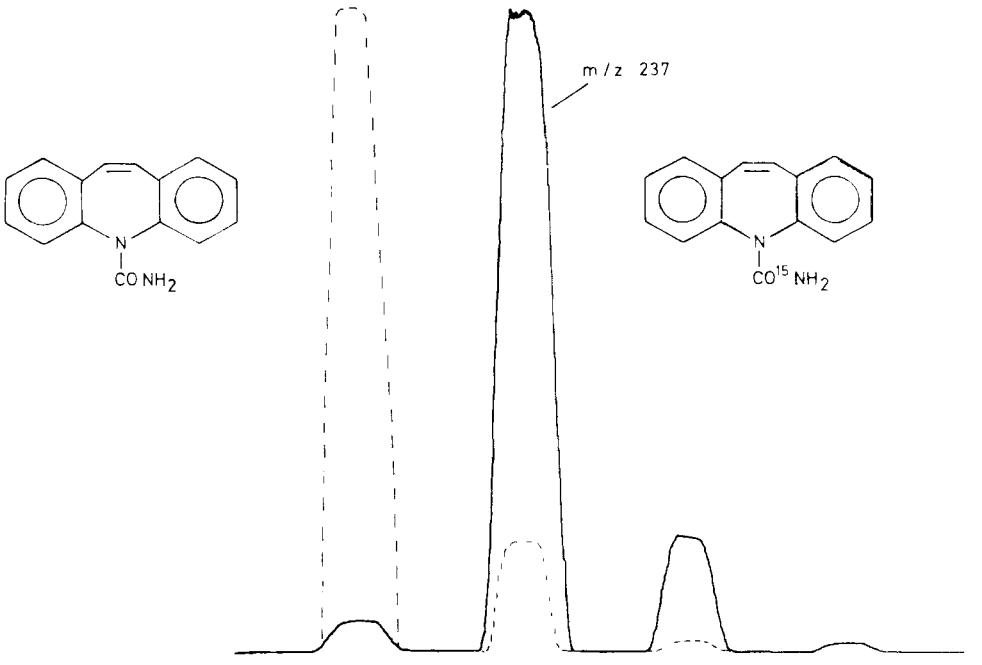

Fig. 2. Comparison of the isotopic distribution of the unlabeled carbamazepine and the ^{15}N labeled drug.

--- calculated natural abundances of the unlabeled drug;

—— mass spectrometric measured isotopic pattern of the ^{15}N labeled compound.

carboxamide group could be determined. The isotopic analysis shows only small protonation which can be neglected in calculating the label content of the drug. A comparison of the measured isotopic distribution of ^{15}N carbamazepine with the theoretical abundances of the unlabeled compound gave a value of 94.98 ±0.5% for the ^{15}N content of the drug (Figure 2). This result is in good agreement with the value of 94.95% found by v. Unruh et. al. using low voltage EI mass spectrometry [7].

Cyclophosphamide, (N,N-bis (chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphorine-2-amine 2-oxide, CP), probably the most widely used drug for the treatment of malignant tumors can be quantitatively determined by mass spectrometry using stable-isotope dilution analysis [8-11]. For quantitation of CP and one of its metabolites 4-ketocyclophosphamide (4-keto-2-bis(2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphorine-2-amine 2-oxide, 4-keto-CP), deuterated analogs, CP-d_{10} and 4-keto-CP-d_6 have been used. The information about the label content of these compounds is a decisive prerequisite for obtaining reliable quantitative data. Isotopic analysis of CP-d_0 and 4-keto-CP-d_0 showed to some extent hydrogen elimination ($[M-1]^+$ ion) and protonation of the molecules ($[M+H]^+$ ions), caused by surface reactions on the FD emitter. The elimination of hydrogen is slightly dependent on the emitter temperature. This effect has to be considered at most with 1% and can be neglected for calculation of the isotopic abundances of the unlabeled compound. The protonation, however, is about 18% for CP between 10-20 mA emitter heating current (e.h.c.). On the assumption that under the same experimental conditions (same emitter, solvent and e.h.c.) protonation of CP-d_{10} (4-keto-CP-d_6) takes place in the same order as with CP-d_0 (4-keto-CP-d_0), the label content of the deuterated analog was calculated:

Cyclophosphamide:	CP-d_8	CP-d_9	CP-d_{10}
	2	18	100

4-keto-Cyclophosphamide:	4-keto-CP-d_5	4-keto-CP-d_6
	17	100

These data confirm results found by Jarman et. al. by direct insertion EI mass spectrometry [12]. The isotopic pattern in FD MS in a mixture of CP-d_0 and CP-d_{10} is shown in Figure 3 .

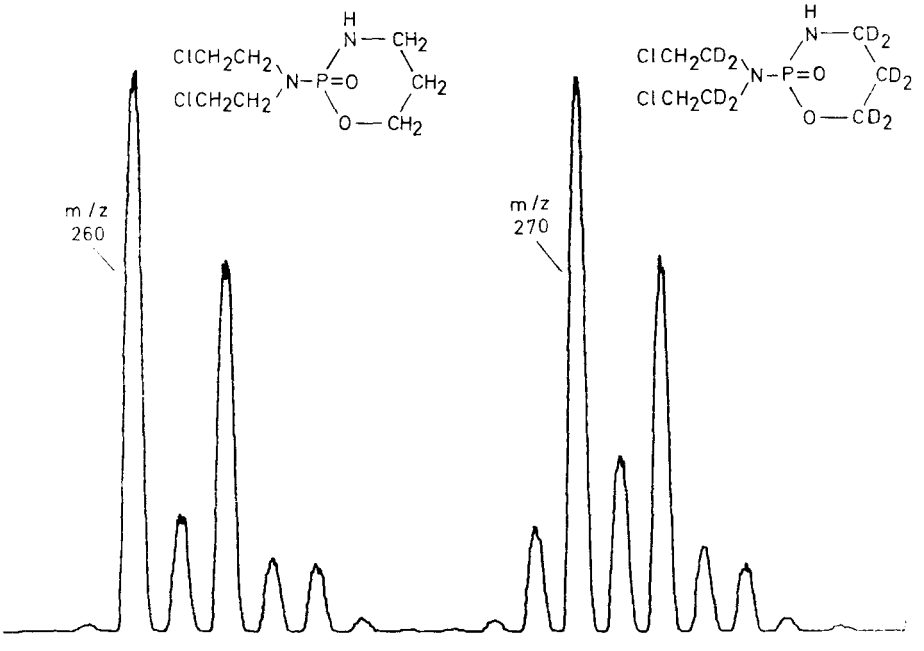

Fig. 3 . Molecular ion group of a mixture of cyclophosphamide-d_0 and cyclophosphamide-d_{10} determined by FD MS.

In desorbing the mixture of both compounds the degree of protonation can be derived from the known pattern of CP-d_0 . Taking this

effect into account the isotopic distribution of deuterium in CP-d_{10} is calculated accordingly. The time required for one analysis is only a few minutes including transfer of the sample and obtaining the plot of the multichannel analyser output.

EXPERIMENTAL

The mass spectrometric investigations have been performed on a commercial double focusing mass spectrometer of type Varian 731 with combined EI/FI/FD source, which was equipped with the Varian SS 200 data system. The anode, a high-temperature activated tungsten wire was on +8 kV and the slotted counter electrode on -3 kV. The ion signals were electrically recorded and accumulated on a multichannel analyser, type Tracor Northern NS-570 A, which was triggered externally by the cyclic magnetic scan of the mass spectrometer. For an accumulation of about 50 scans, a few microliter of sample solution, corresponding to a sample amount of 1-5 ng, are sufficient. The theoretical values for the isotopic abundances of the unlabeled compounds were determined by use of a modified computer program "Isotope" which we gratefully received in the original version from Dr. H.M. Schiebel, Technische Universität Braunschweig.

The compounds investigated have the following origin:

1. Cyclophosphamide and 4-keto-cyclophosphamide, ASTA-Werke, Brackwede;
2. Cyclophosphamide-d_{10}, we are grateful to Dr. M. Jarman, Chester Beatty Institute, Institute of Cancer Research, London, for this compound;
3. Antipyrine, the drug standard, Institute of Pharmacy, University of Bonn;
4. Antipyrine ^{13}C, we thank Prof. Dr. Helge, Kinderklinik Berlin, for the generous gift of this sample;
5. Carbamazepine and the ^{15}N labeled analog, we are grateful to

Dr. Eichelbaum, Medizinische Universitäts-Klinik, Bonn, for obtaining this sample.

CONCLUSION

The results described above show that the FD technique yields mass spectra of high precision and allows to calculate the label contents of drug analogs with an accuracy below 1%. Thus, the capacity to determine the isotopic distribution in labeled drugs prior to biochemical and medical assays is demonstrated. The accuracy of the FD results was ascertained by comparison with other methods such as low energy electron impact MS. Further strong evidence for the quality of the FD values gives the agreement with results detected by the highly accurate combustion analysis [13].

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft and the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen. We thank R. Müller, Universität Bonn for his skilful technical assistance and Dr. W.D. Lehmann, Universitätskrankenhaus-Eppendorf, Hamburg, for his critical comments.

REFERENCES

1. Gaffney T.E., Knapp D., Walle T.- South. Med. J. 67: 990 (1974)
2. Beckey H.D. and Schulten H.-R. - Angew. Chem. 87: 425 (1975); Angew. Chem. Int. Ed. Engl. 14: 403 (1975); Schulten H.-R.- in Methods of Biochemical Analysis, Glick D., Ed., Wiley Interscience, New York, Vol.24: 313 (1977); Schulten H.-R.- Int. J. Mass Spectrom. Ion Phys. 32: 97 (1979)
3. Beckey H.D.- Principles of Field Ionisation and Field Desorption Mass Spectrometry, Pergamon Press, Oxford,(1977)
4. Lehmann W.D, Schulten H.-R. and Schiebel H.M.- Z. Anal. Chem. 289: 11 (1978)
5. Helge H., Gregg G., Jäger E., Knies S., Bochert G., and Nigham S. in Stable Isotopes: Proceedings of the Third International

Conference, Klein E.R. and Klein P.D., Eds., Academic Press, New York, 495 (1979)

6. Seelmann-Eggebert W., Pfennig G. and Münzel H.- Karlsruher Nuklidkarte, Kernforschungszentrum Karlsruhe (1974)
7. Unruh v. G.E., Köthe K.W. and Eichelbaum M.- in Stable Isotopes: Proceedings of the Third International Conference, Klein E.R. and Klein P.D., Eds., Academic Press, New York, 453 (1979)
8. Schulten H.-R. - Biomed. Mass Spectrom. 1: 223 (1974)
9. Schulten H.-R. - Cancer Treat. Rep. 60: 501 (1976)
10. Cox P.J., Farmer P.B., Foster A.B., Gilby E.D. and Jarman M.- Cancer Treat. Rep. 60: 483 (1976)
11. Lehmann W.D. and Schulten H.-R. - Z. Anal. Chem. 290: 121 (1978)
12. Schulten H.-R., Lehmann W.D. and Jarman M.- in Quantitative Mass Spectrometry in Life Sciences, de Leenheer A.P. and Roncucci R.R., Eds., Elsevier, Amsterdam, 177 (1977)
13. Schiebel H.M. and Schulten H.-R. - Naturwissenschaften 67: XXX (1980)
14. Achenbach C., Ziskoven R., Köhler F., Bahr U. and Schulten H.-R. - Angew. Chem. 91: 944 (1979); Angew. Chem. Int. Ed. Engl. 18: 882 (1979)