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Note

Isotope cluster chromatography to locate isotopically labeled species

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The metabolic fate and pharmacokinetics of drugs are conveniently studied by gas chromatography—mass spectrometry (GC—MS). However, because body fluid samples are complex mixtures, it is frequently difficult to confidently identify metabolites versus non-metabolites. Many investigators have resolved this problem by the use of stable-isotope labeled analogues in conjunction with the non-labeled drug [1]. The incorporation of ^{2}H, ^{13}C, ^{15}N or ^{18}O into the parent drug and subsequently into its metabolites reliably distinguishes products derived from the drug from normal constituents of body fluids. No radioactivity is involved, and there is no break in the administration of the drug, so risks to the patient or experimental animal are minimal. If a mixture of stable isotope labeled and non-labeled drug is administered, the mass spectra derived from such a mixture, or from metabolites of such a mixture, will display characteristic isotopic doublets, unambiguously identifying compounds which originated from the drug.

Deuterium, because it is relatively inexpensive and easy to incorporate into molecules, has been the most widely used of the stable isotopes. Deuterium labeled analogues have aided in studies of the metabolism of propoxyphene [2], phencyclidine [3], warfarin [4], pyrazole [5], and alprenolol [6], to name a few. Compounds discovered in blood or urine samples which display mass spectra with the appropriately spaced isotope doublets are presumed to have come from the drug. The GC—MS analysis of these samples involves the scan-by-scan searching of hundreds of mass spectra, seeking those which show the characteristic pattern of ions.

Recently, we have introduced a computer technique which would be of great assistance in such a search. Isotope cluster chromatography [7] was designed to search GC—MS data sets for mass spectra displaying the isotope cluster patterns resulting from chlorine and/or bromine atoms. These elements have two

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abundant, naturally-occurring stable isotopes; and their mass spectra are very distinctive. We have now expanded that computer program to allow the investigator to specify any patterns of ions for which to search. We report here several potential applications of isotope cluster chromatography to these artificial isotope patterns. In addition to drug metabolism studies, the technique can be used in experiments in which a mixture of stable isotope labeled and non-labeled derivatizing reagents is used. In this case, the computer assists in locating the spectra of compounds which contain a particular functional group which has been derivatized by distinguishing such compounds from others which do not display the isotopic doublet.

MATERIALS AND METHODS

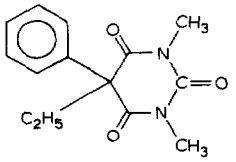
Unless specifically stated, all solvents and reagents were obtained from commercial sources and were used without further purification. [$^2H_4$]Methanol (99.5% isotopically pure) was purchased from Alfa-Ventron (Beverly, MA, U.S.A.).

Methanolic hydrochloric acid was prepared by bubbling dry hydrogen chloride gas through methanol. The molarity was determined by titration against standard sodium hydroxide solution, and was adjusted to approximately 3 *M* hydrochloric acid. The dimethylphenobarbital, stable-isotope labeled dimethylphenobarbital, and spiked serum samples were generous gifts of Dr. Agnes Van Langenhove of M.I.T. (Boston, MA, U.S.A.).

Methyl esters were prepared by dissolving the corresponding carboxylic acid in 200—400 μl of [$^2H_4$]methanol and adding an equivalent amount of 3 *M* methanolic hydrochloric acid. The reaction was conducted at room temperature for 45 min. Excess reagents were removed under a stream of dry nitrogen.

GC—MS analyses were performed on a Hewlett-Packard 5985B system. Chromatography was on a 30 m × 0.32 mm fused silica capillary, wall coated with Durabond DB-1 (J&W Scientific, Orangevale, CA, U.S.A.). The splitless injector and transfer lines to the mass spectrometer were maintained at or above 250°C. Helium at a flow-rate of 1—2 ml/min was used as carrier gas. The column conditions were as indicated in figure legends. The mass spectrometer ion source was at 200°C, and spectra were continuously scanned approximately every 2 sec. The electron energy was 70 eV.

Computer programs were written in BASIC with FORTRAN subroutines; and listings are available from the author. The user enters the relative intensities of the isotope pattern for which he/she is searching. An unknown mass spectrum is broken down into a series of parts, each containing exactly as many masses as are present in the known isotope pattern. Each of these parts is then compared to the known cluster to see if it is similar. A reverse library search-type algorithm is used to determine similarity, as in our previous work [7]. A total score for the unknown mass spectrum is calculated as a weighted sum of all of the similarity indices. When each spectrum in a GC—MS data set has been searched and assigned a score, a plot of score versus scan number produces a chromatogram such as that in Fig. 2a or Fig. 3a.



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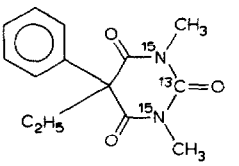

II

RESULTS AND DISCUSSION

The mass spectrum of a mixture of dimethylphenobarbital (I) and dimethyl-[^{13}C,$^{15}N_2$]phenobarbital (II) is shown in Fig. 1. The ion doublets separated by 3 atomic mass units (a.m.u.) at *m/z* 232—235, *m/z* 245—248, and *m/z* 260—263 (shown in boldface type in Fig. 1) are a result of a nearly equimolar mixture of the non-labeled and stable-isotope labeled compounds [8]. This pattern will appear in the mass spectra of the parent drug and any metabolites which retain the stable-isotope label, so it is this pattern which the computer is asked to seek. The user can input any isotope cluster pattern of up to twenty masses at any relative intensities; but in this case, the investigator would specify a doublet of about equal intensities separated by 3 a.m.u. The computer then conducts a scan-by-scan search of any GC—MS data set for the presence of such clusters, just as in the previous description of isotope cluster chromatography [7]. A score is assigned to each mass spectrum reflecting the probability that such a cluster occurs in the spectrum and the abundance of the ions in potential clusters. A plot of these scores versus scan number produces an isotope cluster chromatogram as shown in Fig. 2a. A serum sample spiked with the mixture of labeled and non-labeled dimethylphenobarbitals was chromato-

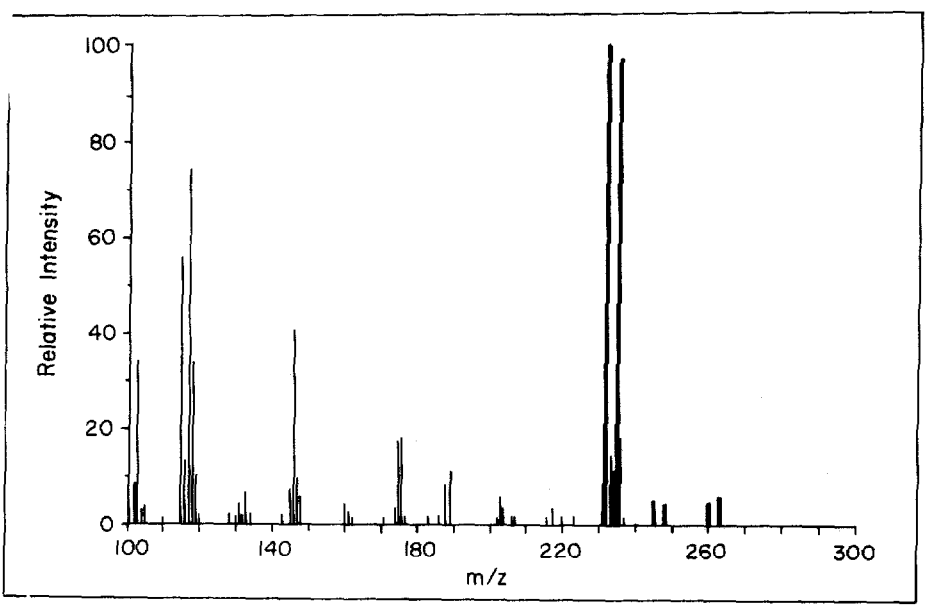

Fig. 1. Mass spectrum of an approximately equimolar mixture of I and II. The ion doublets at *m/z* 232—235, 245—248, and 260—263 are diagnostic (see text) and are highlighted.

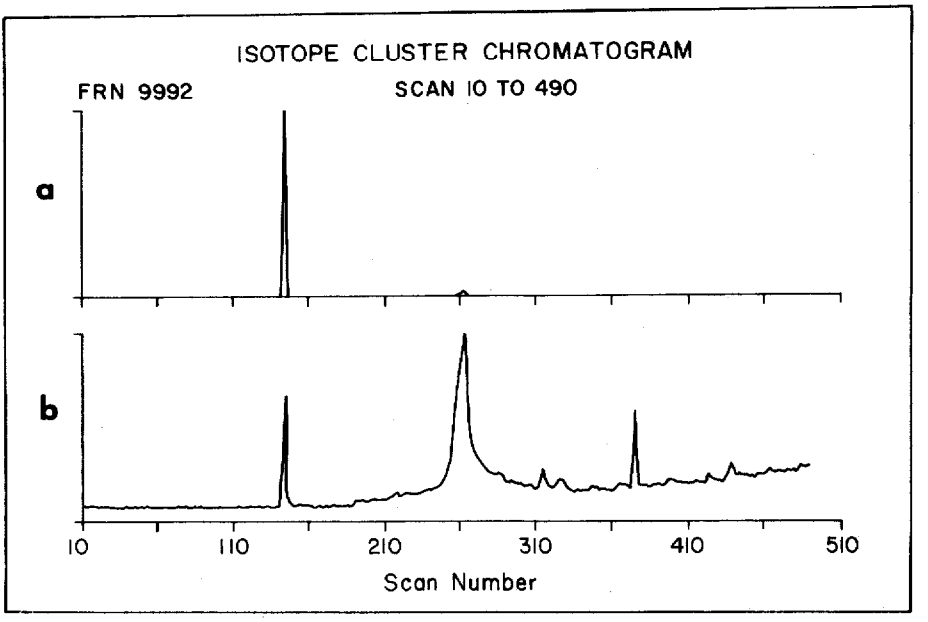

Fig. 2. (a) Isotope cluster chromatogram and (b) total ion profile for the GC—MS analysis of a serum extract spiked with I and II. The GC oven temperature was held at 120°C for 2 min, and then linearly programmed at 10°C/min to 330°C.

graphed and searched. Only one peak, around scan 145, shows a maximum in the isotope cluster chromatogram, indicating an intense doublet of the type sought. The peak corresponds to the expected retention time of dimethyl phenobarbital. If metabolites had been present and had contained the isotope doublet, they would also have been found. Mass chromatograms [9], a useful alternative data reduction method, can only be used in cases where the mass spectrum or structure (and hence, a characteristic ion) is known. The isotope cluster chromatogram locates all metabolites which display the appropriate isotope pattern, regardless of their structure.

Drug metabolism is not the only area of applicability of such a technique. Nau and Riordan [10] have used $H_2{}^{18}O$ to probe the mechanisms and active sites of enzymes. Isotope clusters produced by the incorporation of ^{18}O into peptides could also be located by computer.

There are two phenomena which could possibly distort the isotope patterns observed in the mass spectra of drug metabolites. Mimura and Baba [11] have observed that in the metabolism of the drug, paeonol, an isotope effect causes the non-labeled and stable-isotope labeled drugs to be metabolized at different rates. Thus the isotope cluster of the metabolite is no longer exactly the same as in the original dose mixture. Such an effect is occasionally observed with deuterium labeled compounds, but rarely with ^{13}C, ^{15}N, or ^{18}O.

A second concern is that the stable-isotope labeled and non-labeled compounds may be at least partially separated chromatographically. As a result, in any mass spectrum, the isotope pattern observed would also reflect this separation. Again, the effect is most pronounced with deuterium labeling, particularly in compounds with several deuterium atoms. In our experience,

working with di- and trideutero compounds, we have noticed a slight distortion of the isotope pattern on the leading and trailing edges of a chromatographic peak; but in the peak center, the isotope ratios are as would be predicted. This is consistent with the observations of other workers [11], and has not imposed a serious limitation on the program thus far.

The time required to generate the isotope cluster chromatogram in Fig. 2a was about 5 min to search 480 mass spectra for isotope clusters. During this time, the computer is doing its calculations without user interaction and can be left unattended. Without this data reduction by computer, the analyst would be faced with the task of looking at each of the 480 mass spectra individually, and assessing in each spectrum the likelihood that isotope clusters are present. Clearly a dramatic savings in time and effort can be realized.

Another area of application of this stable-isotope labeling technique is in the formation of chemical derivatives. Methylation [12] and acetylation [13] using mixtures of non-labeled and stable-isotope labeled reagents have been reported. These derivatives produce mass spectra with characteristic isotope patterns which can then be used to identify compounds containing a reactive functional group or to aid in the determination of fragmentation mechanisms. The isotope cluster chromatography program described above can be used to locate these diagnostic isotope clusters in a GC—MS data set as well.

As a simple example, a mixture of compounds including one carboxylic acid was prepared. When the mixture was methylated with methanolic hydrochloric acid including both [$^1H_4$]methanol and [$^2H_4$]methanol, only those compounds capable of being methylated would incorporate the stable isotope label. In this case, the non-labeled and labeled methanol were in a ratio of

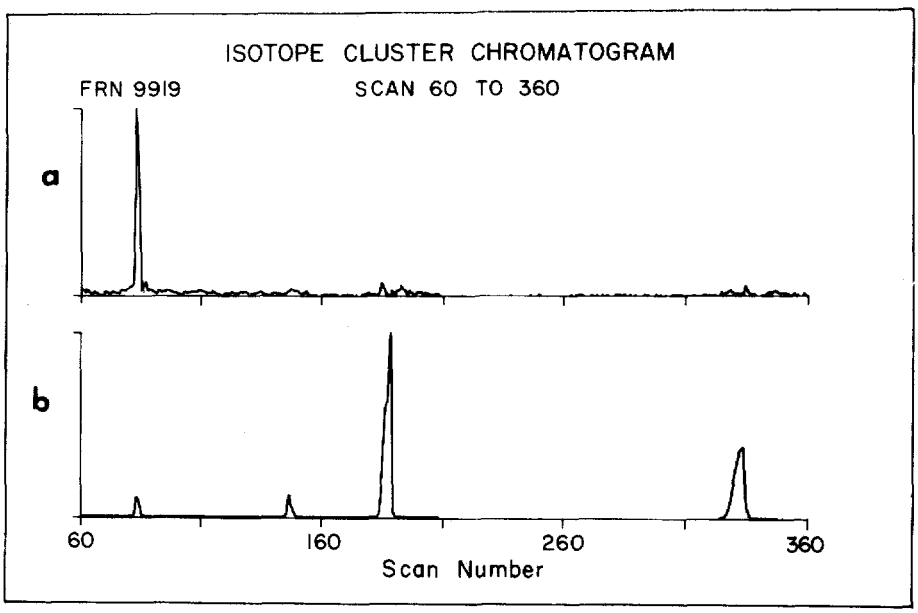

Fig. 3. (a) Isotope cluster chromatogram and (b) total ion profile for the GC—MS analysis of a sample methylated with a mixture of [$^1H_4$]- and [$^2H_4$]methanolic hydrochloric acid. The GC oven temperature was linearly programmed from 100°C to 330°C at 16°C/min.

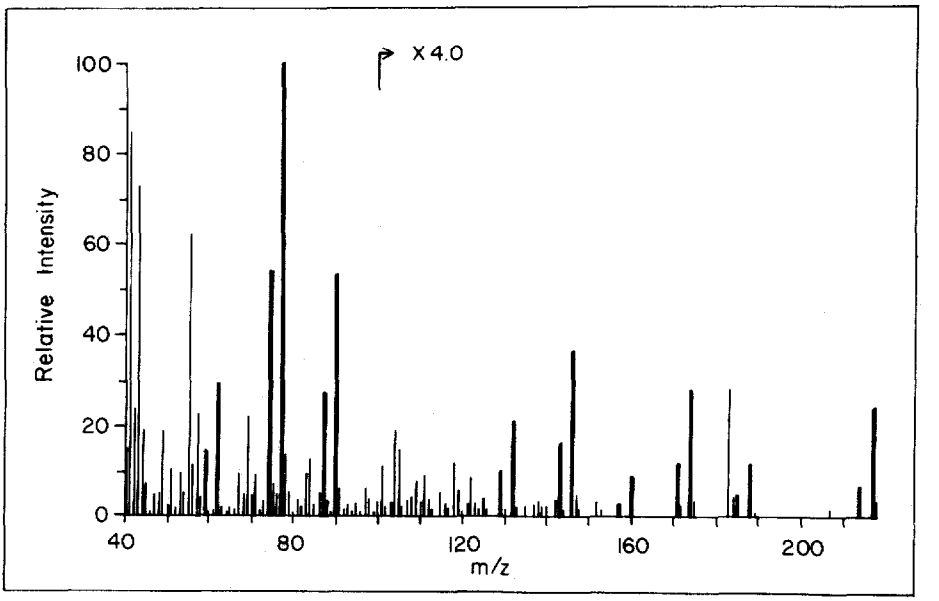

Fig. 4. Mass spectrum of a mixture of [$^1H_3$]- and [$^2H_3$]methyl laurate from scan 83 of Fig. 3. Ion doublets characteristic of the non-label/stable-isotope label (in a ratio of about 1:2) are highlighted.

about 1:2, so compounds which were methylated would show a doublet, separated by 3 a.m.u. in a ratio of about 1:2. This information is given to the computer, which searches the GC—MS data set for the appropriate isotope cluster, and generates the plot shown in Fig. 3a. One peak appears around scan 83 and its mass spectrum is shown in Fig. 4. The isotope doublets are shown in boldface type as before. The spectrum is that of methyl laurate which has been stable-isotope labeled. The mass chromatogram of *m/z* 74 could have been used to locate the methyl laurate as well, since the *m/z* 74 ion is characteristic of saturated fatty acid methyl esters [14]. However, isotope cluster chromatography is a more general technique since it could also locate methyl esters of compounds which do not display a prominent ion at *m/z* 74, such as benzoic acid derivatives.

In summary, the technique of isotope cluster chromatography has been expanded to include a search for any isotope cluster specified by the user. The products of metabolic or chemical reactions can be quickly located by computer, allowing the investigator to concentrate his/her time on those compounds which have incorporated a stable-isotope label. Although the data presented were all collected as positive ions in the electron ionization mode, the technique would work equally well for chemical ionization, and for negative ion mass spectra, provided only that the isotope patterns remain undistorted.

ACKNOWLEDGEMENTS

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