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A STABLE ISOTOPE RATIOMETER-MULTIPLE ION DETECTOR (SIRMID) UNIT FOR QUANTITATIVE AND QUALITATIVE STABLE ISOTOPE STUDIES BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

by

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Abstract

We have designed and constructed a stable isotope ratiometer-multiple ion detector (SIRMID) unit which can drive existing gas chromatograph-quadrupole or magnetic sector mass spectrometers to monitor up to six ions in turn. Each of the three pairs of ions can be selected for quantitation; thus three different or successive components can be analysed in a single GC run. A background subtraction option permits the ion intensity in the absence of sample to be subtracted automatically during sample measurement. Displays of accumulated counts and isotope ratio are updated twice per second during the measurement and can be printed out at its conclusion. All six ions can be monitored in the analog mode by parallel outputs to a multipen recorder. Experience gained in the construction of this prototype indicates that SIRMID units could be commercially available for $10K, or about 1/3rd to 1/6th of the cost of even an inexpensive computer system.

The use of GC/MS in clinical pharmacology and in kinetic, metabolic and diagnostic applications of stable isotopes often requires repetitive examination and quantitation of selected ions in the mass spectra of gas chromatographic effluents. Switching between 2-8 ions in a mass spectrometer, often called multiple ion detection (MID) (1,2) can be achieved by accelerating voltage alternation (3,4) in a magnetic sector instrument or by manipulation of the dc voltage in a quadrupole mass filter (5,6); a number of procedures and devices have been described and are available for several commercial instruments. Quantitation of ion intensity can be accomplished by recording the ion envelopes of the gas chromatographic peak on multi-pen recorders and measuring the peak heights, or by direct measurement of the ion currents during the gas chromatographic peak with a computer peak acquisition system. The former method requires hand calculation of analog data, while the latter method requires a substantial capital investment. Between these two extremes, there appears to be a requirement for an inexpensive GC/MS accessory that would provide the combined capability of a stable isotope ratiometer and a multiple ion detector. Commercial availability of such an accessory would permit many GC/MS systems already in use to be employed in the types of applications described above, and would facilitate the wider use of stable, non-radioactive tracers in pediatric and obstetric patients. We describe here the design features, operation and performance of such a stable isotope ratiometer-multiple ion detector (SIRMID) as constructed in our

laboratory and evaluate the experience gained from this prototype from the standpoint of the design of a commercial unit.

SIRMID: Instrumental Design, Construction, Operation and Use

Design objectives. After three years experience with the system previously described at this Symposium, we selected the following features to be incorporated into a new low cost instrument chassis: 1) the ability to monitor up to 6 ions in cyclic manner; 2) the ability to carry out accurate quantitation on two ions at a time; 3) provision of all computations involved in the calculation of isotope ratios including any differential amplification factors, and the subtraction of background ion intensities, terminating in a printed output of isotopic ratios; 4) the ability to make isotope ratio measurements on three gas chromatographic components within a single chromatogram and 5) the capability of being used on either a magnetic sector or quadrupole mass spectrometer.

Instrument construction. The SIRMID unit was constructed by the Electronics Division at Argonne National Laboratory and employs integrated circuit technology to achieve low power requirements with high noise rejection capabilities. The SIRMID construction includes the use of a calculator chip and memory unit for temporary storage of data and all controls, data accumulation and calculations employ binary logic. On the front panel are six ion modules, providing the ability to monitor up to six ions or to provide three ion pairs for ion quantitation. Each module has a horizontal row of eight

digital thumbwheels; four are used for focussing the instrument to the nearest 0.1 mass unit, four provide digital control of the amplification of the electron multiplier output for that ion. When operated in conjunction with a quadrupole mass spectrometer, the digital value of the focus portion reads directly in mass units, and sets the level on a digital to analogue (D/A) dc power supply establishing the ion focus. When used in conjunction with a magnetic sector instrument, the digital settings correspond to the ratio: lower mass/upper mass and the D/A power supply decrements the accelerating voltage to achieve the desired focus. The ion amplification is through a 12 bit digital amplifier, providing a range of 1:4000. A selector switch permits focussing on either member of a given pair and in the central position alternates the focus automatically. A sweep control provides a ramp voltage covering a 10 amu range to permit location of the desired ion and through a ten-turn Helipot, may be reduced in a continuous manner from 10 to 0 amu. The sweep is symmetrically centered on the ion of interest, so that as the sweep width is narrowed, the ion remains in the center of the sweep display. Beside each ion module is a light-emitting diode with the designation OVLD. If the diode lights as the ion of interest is scanned, it indicates that the signal has exceeded the 10 v limit of the analogue to digital converter and that counts are being lost. Finally, a CHANNEL SELECT switch permits selection of the desired ion pair for quantitation.

Ion quantitation is carried out during the sweep across the ion peak. A 10 bit D/A converter provides a ramp voltage with 1024 steps

of equal size. At each step the ion current intensity is measured by a 14 bit A/D converter, which achieves a resolution of 1 part in 16384 for a 10 volt full scale input. Each sweep is completed in 200 ms and the focus is shifted to the other ion; the total cycle time, including 50 ms settling time, is thus 500 ms and each ion can be quantitated twice per second. The counts accumulated during each sweep are stored in the appropriate ion register for display in two 6 digit light-emitting diode displays. A pre-scaling factor of 2^{10} to 2^{14} can be selected to reduce the magnitude of the ion count measurement. A third 6 digit register in the display can be selected to show either elapsed time to 0.01 seconds or to indicate the isotope ratio to four places. The isotope ratio display is updated after every pair of sweeps, or twice per second, and is calculated from the accumulated net counts of Ion 1 and 2, and the amplification factors set in each of the ion modules.

A background subtraction capability is present in the SIRMID unit which makes it possible to determine the ion intensity at the selected mass in the absence of sample and correct the accumulated counts to reflect net counts on a live time basis. A selector switch permits background ion intensity to be accumulated for 2^7 sweeps, equivalent to 2^{17} A/D conversions. By pressing BKG STORE, this value is recorded in memory, and the quantity representing the time-averaged background ion intensity per conversion is subtracted after each conversion step in the sweep ramp.

Analog monitoring of each ion is provided by routing the output of the 512th A/D conversion (presumptively the center of the ion peak)

to a D/A converter. If the ion is not being quantitated, the program steps directly to the 512th step in the ramp in order to sample the ion intensity. This requires only 100 ms per ion and thus the total cycle time is 250+250+100+100+100+100 or 900 ms to quantitate two ions and track all six by analog monitoring. A selector switch permits either 2 or 6 ions to be monitored; the former is used when repetitive quantitation of a given ion pair is desired. Individual BNC connectors for the ions and their isotopic ratios are provided for cable connection to a multi pen recorder.

A six digit line printer, operated by push button, prints the displayed information at a rate of 1 line/second. A volt meter with ranges of 10 v, 1 v and 50 mv permits zeroing of the electron multiplier input, and a single trim pot adjusts any internal offset within the A/D converter.

Instrument operation. To operate the SIRMID unit, the mass spectrometer is first focussed on the ions of interest by introducing the appropriate standards via probe, or as a gas chromatographic sample. With the SWEEP control at its maximum setting, the sweep range covers 10 amu, and the whole mass number desired is set by the digital thumb wheels. When the sweep range has been narrowed to 1 amu, trimming of the mass setting by the 0.1 amu thumbwheel achieves final focus and brackets a single ion within the channel. The display registers are cleared and background is counted for each pair of ions to be determined. By pushing BKG STORE, these values are transferred into the memory and recorded; additionally, they may be printed out on the lister by pressing PRINT. Next, the

sample is introduced, either by probe or as a gas chromatographic sample and as soon as the ion intensity begins to increase, the operation mode switch is turned to START. When the evolution of the sample from the probe has ceased, or the gas chromatographic peak has been eluted and the ion intensity has returned to baseline, the mode switch is turned to PAUSE. This permits the clock to continue running, in the event that the retention time of a gas chromatographic run is being monitored, but terminates ion quantitation. (Because the display mode shows net counts accumulating, it is quite easy to determine when quantitation can be terminated without significant loss of information: the counts displayed change at an ever decreasing range, until they are varying only in the fourth or fifth place). Pressing the PRINT button lists the accumulated counts for Ion 1, Ion 2 and the isotope ratio, corrected for attentuation settings. Alternatively, the ion counts and elapsed time may be printed by switching the display mode from ISOTOPE RATIO to TIME. The display registers for the ion counts are now cleared (the TIME display is cleared only with the instrument mode switch at STOP) and the CHANNEL SELECT switch is set to the ion pair to be quantitated in the next gas chromatographic peak. This automatically establishes the new ion focus and simultaneously introduces the new attenuation factors and background subtraction values into the ion intensity and isotope ratio calculations. The instrument is now ready for the next measurement; typically, data may be printed, display registers cleared and channel selection altered within 10 seconds or less,

thus permitting measurements on closely-spaced gas chromatographic peaks in rapid succession.

Isotope Dilution Measurements. Two dilution series were constructed to test the ability of the SIRMID unit to measure isotopic ratios over a wide range of isotope concentration. In the first instance, singly labeled ^{15}N urea and unlabeled urea were used to construct a series ranging from 10% to 0.05% ^{15}N content. Aliquots of the methanol solutions were evaporated in probe tubes and introduced through the probe system into the mass spectrometer. The source temperature was 125°C and the reagent gas was methane at 1 torr. Isotopic composition was determined from the ratio of ion intensities at m/e 62 to m/e 61. In the second instance, a dilution series was prepared, using 24-^{13}C chenodeoxycholic acid as the labeled species in concentrations from 50% to 0.1%. Aliquots were derivatized to form the methyl ester acetates, and 3 µg samples were injected on an 0.25% SP 525 column at 250°C in the Biospect instrument. Isotopic composition was determined from the ratio of m/e 372 to m/e 371.

The effect of sample size on the determination of isotopic content was examined by determining the proportion of molecules containing 1, 2 or 3 ^{13}C atoms in methyl stearate samples by probe or by gas chromatographic introduction. This provides an internal series of isotope ratios of approximate 20%, 2% and 0.2%, and samples from 1 µg to 90 pg were run in quintuplicate.

Multiple Ion Detection and Serial Isotope Ratio Measurements. To illustrate the use of the SIRMID unit both as a multiple ion

detector and in the sequential measurement of isotope ratios in successive peaks in a gas chromatogram, the instrument was set to monitor m/e 373, m/e 371 and m/e 369. This provided a record of the elution of the monohydroxy bile acid, lithocholic; the dihydroxy bile acids, deoxycholic and chenodeoxycholic; and the trihydroxy bile acid, cholic acid, when this mixture of bile acids was chromatographed as the methyl ester acetates. In each instance, the next higher mass unit was also paired with the ion used for detection, to determine the proportion of molecules containing a single atom of ^{13}C. As in the methyl stearate, this proportion may be predicted from the natural abundance of ^{13}C and amounts to 1.1% x the number of carbon atoms in the ion. These measurements thus provide a test of the ability of the SIRMID to determine four isotopic ratios in succession within the same gas chromatographic run. Background was measured and stored for each pair of ions before injection of the sample; from the multi-pen recorder the start and conclusion of each peak could be seen, and after printout of the data, the next pair of ions to be quantitated could be selected by setting the channel selector switch. Five consecutive runs were made to provide estimates of the variability of data obtained in this manner.

Results and Discussion

In the 15 months that the SIRMID unit has been in service, it has proved to be rugged, simple to operate and a significant improvement over systems built earlier in our laboratory. The ability to

measure isotope ratios over a wide range of isotope ratios over a wide range of isotopic content is shown in Figure 1 and 2, representing the dilution series for urea and chenodeoxycholic acid. The standard deviations of the points in these series are typically within the diameter of the circles used to represent them. In the urea study, a single isotopic label as ^{15}N could be detected at concentrations as low as 0.05%; in samples of 600 ng, this represents an equivalent detection limit of 300 pg for the ^{15}N form. The larger molecule, chenodeoxycholic acid, containing 24 carbons and labeled in the carboxyl group with ^{13}C, could still be quantitated when it was present at 0.5%, where it represented 15 ng in the 3 ug sample. The departure from linearity at the lower ends of both series does not appear to be the result of improper quantitation, but rather from the limitations of a quadrupole mass filter and its inability to prevent small perturbations of ion resonance. These perturbations, which may be caused by trace impurities on the surfaces of the quadrupole rods, result in ions "spraying" into other mass trajectories and being collected at inappropriate mass positions. This may place a practical limitation on the dynamic range of isotopic ratios which can be determined using a quadrupole mass spectrometer. We plan to examine the dilution range of the chenodeoxycholic acid ^{13}C on the Perkin Elmer DF 270 magnetic mass spectrometer with the SIRMID to establish whether or not the higher accelerating voltage of the magnetic instrument insures a more rigid segregation of ion species in the mass analyzer.

A more practical problem, involving isotopic ratios more likely to be encountered in inverse isotope dilution experiments, is examined in the methyl stearate studies shown in Figure 3. These are concerned with the instrument's ability to measure the same isotopic ratio over a wide range of sample size, and they show that the SIRMID unit yielded isotope ratios of acceptable accuracy on sample sizes as small as 90 pg. The M+1 abundance is approximately 20% of the molecular ion M. The isotopic ratios obtained on the 90 pg sample thus represent measurement of the small component at the 20 pg level, and the standard deviation of the measurements was 0.27 pg. Although the sensitivity of the mass spectrometer ultimately determines the limits at which an isotopic species can be quantitated, some calculations of the ion quantitation capability of the SIRMID unit may be of interest. If the minimum signal detected by the A/D converter is taken to be 1 bit/conversion (equivalent to 1 count stored in the ion register), this corresponds to 625 μV for the duration of the conversion, and if continued for 1 second, would yield 2 x 1024, or 2048 counts for that ion. The electron multiplier gain is typically 10^5 and the electrometer gain following it, at midrange, has a setting of 2×10^7; thus the current required to produce this signal is $(625 \times 10^{-6})/(2 \times 10^7 \times 10^5)$ or 3.12×10^{-16}A. Dividing by coulombs/charge (1.6×10^{-19}) gives a value of 1900 ions/second for the minimum count rate of 2048/second or about 1 count/ion. Thus with the range

of gain settings available (1×10^5 to 5×10^9) ion quantitation, the SIRMID unit can produce from 0.05 to 262 counts/ion. Two practical considerations suggest that the full range may not be necessary, even for optimum use of the SIRMID unit: first, the gain factor of the electron multiplier changes with age and its value would have to be determined at periodic intervals to establish the true conversion rate between ions and counts; second, no improvement in counting statistics accrues from a count rate in excess of 1 count per ion. As long as the conversion rate is approximately 1, the isotopic ratio measurements will have the maximum precision afforded by the ion statistics.

The use of the SIRMID unit in a multiple ion detection mode is illustrated in Figure 3, which illustrates the individual ion records for the mono-, di- and trihydroxy bile acid when chromatographed as their methyl ester acetate derivatives. This mode has proved to be highly valuable in qualitative identifications of bile acids by chemical ionization mass spectrometry and for monitoring the emergence of specific ion peaks of interest. For aesthetic reasons however, the use of a A/D-D/A system is not entirely satisfactory because at low signal levels, a single bit fluctuation results in quantized pen responses. These produce the "grass" seen in the lower trace of Figure 3. A better alternative might be to use the integral of the sweep instead of the value at the 512th conversion to produce a smoother recorder trace and we are currently exploring this possibility.

The ability to measure isotopic ratios in successive gas chromatographic peaks, such as those in Figure 3, provides versatility, flexibility and increased throughput of samples when more than one measurement is required. Table 1 shows the results of such sequential measurements in five runs of bile acid mixtures. The standard deviation ranged from 0.3-1.2% of the ratio and the absolute agreement of the observed isotope ratio with the theoretical value was excellent for the mono and dihydroxy bile acids. The somewhat larger variability and departure from theoretical abundance for the trihydroxy bile acid, cholic acid is thus genuine, not instrumental in origin, and apparently reflects differences in chemical ionization processes for this bile acid as opposed to the other three.

In the instance of the bile acids, several practical applications of the ability to conduct multiple isotope ratio measurements suggest themselves. For instance, with the availability of bile acids labeled with ^{2}H or ^{13}C (7) it is now feasible to quantitate bile acid composition by inverse isotope dilution techniques in samples which are size limited such as plasma or liver biopsies. Moreover, it is also practical to conduct simultaneous studies of the turnover rates of all four bile acids and thus validate the multi-compartment model proposed by Hoffman and Hofmann (8). Examples from clinical pharmacology such as the determination of neurotransmitter levels, or drug metabolite studies also suggest themselves as having potential application for such instrumentation.

As an advanced prototype for any commercial instrument this SIRMID unit has provided some important guidelines for future versions. It may prove advantageous, for example, to substitute a voltage-to-frequency converter for the expensive 14 bit A/D converter used in the present unit. The opportunities for improvement in the MID portion have already been described and would also benefit from a VFC substitution. Finally, and most significant for the eventual selling cost, the introduction of a microprocessor would substantially reduce production costs by eliminating the hard-wiring constructions employed in this unit, and would provide additional flexibility in the number of ions monitored, ion focussing, and data presentation. We have been advised that an advanced SIRMID design incorporating these and other features is in production by Scientific Research Instruments, Inc., Baltimore, MD., and will be offered for sale in the latter half of 1975[1]. The selling price will be close to our original construction cost of $10K and will thereby provide a substantial analytical capability for isotopic ratios in organic molecules, as well as for multiple ion detection for 1/3 to 1/6 of the cost of a computer data system. Such a capability will, we hope, facilitate and expand the use of stable isotopes in pharmacological and clinical research.

Acknowledgments

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Table I

Determination of the Natural ^{13}C Abundance of Individual Bile Acids in a Mixture Using the SIRMID Unit

Bile Acid:	Lithocholic	Deoxycholic	Chenodeoxycholic	Cholic
Ions Monitored:	374/373	372/371	372/371	370/369
	^{13}C Abundance			
	%	%	%	%
Run 1	27.26	27.42	27.42	28.99
Run 2	27.61	27.54	27.38	28.93
Run 3	27.60	27.60	27.59	28.72
Run 4	27.53	27.60	27.42	29.85
Run 5	27.58	27.59	27.47	29.06
Ave:	27.52	27.55	27.46	29.11
SD	±.15	±.08	±.08	±.43
Theory*	27.75	27.72	27.72	27.68

*Based upon elemental composition of ion

Figure Legends

Figure 1. Isotope dilution measurements of urea-^{15}N by chemical ionization quadrupole mass spectrometry using the SIRMID unit.

Figure 2. Isotope dilution measurements of chenodeoxycholic acid-24-^{13}C by chemical ionization quadrupole mass spectrometry using the SIRMID unit.

Figure 3. Isotope ratio measurements on methyl stearate by chemical ionization mass spectrometry using the SIRMID unit.

Figure 4. Single ion monitoring of mono-, di- and trihydroxy bile acids using the SIRMID unit.

Footnote

[1]Mr. Richard Hall, General Manager, Scientific Research Instruments, Inc., Baltimore, Maryland 21207; personal communication.

Fig. 1

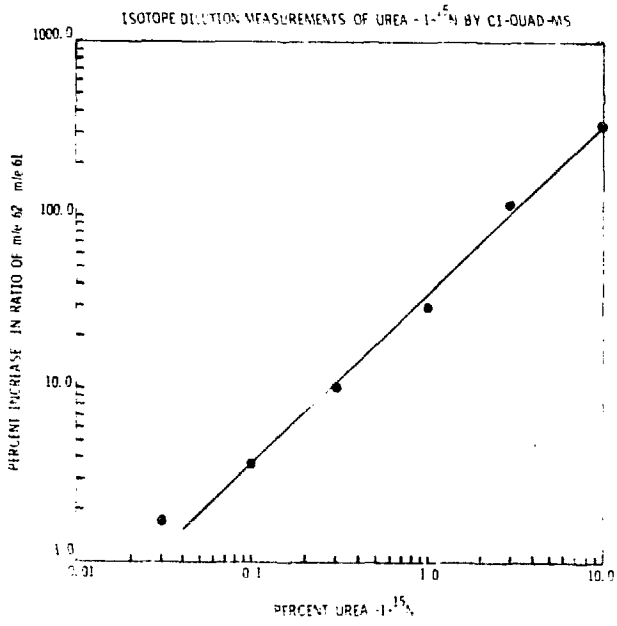
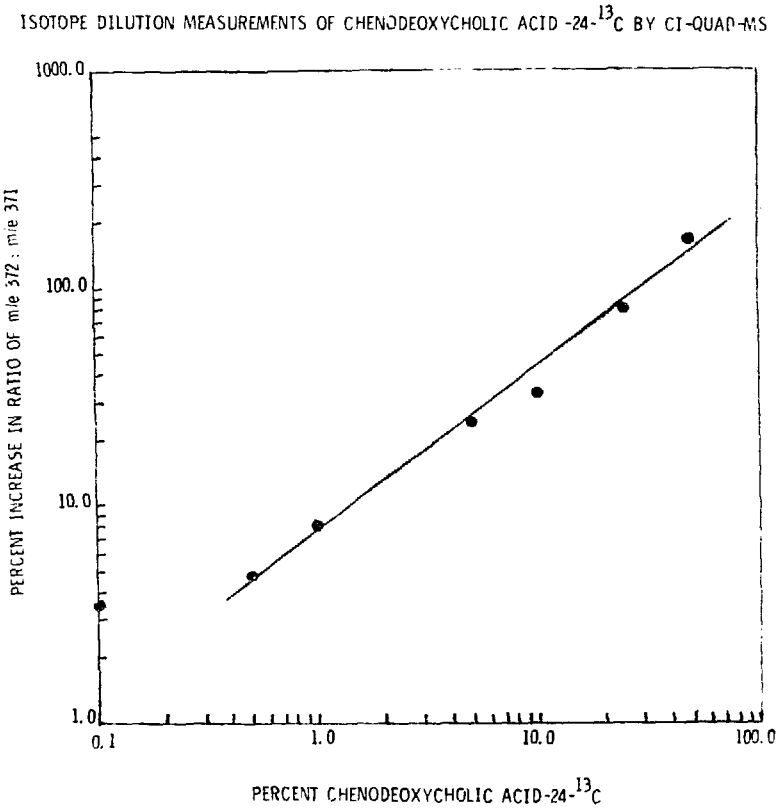

ISOTOPE DILUTION MEASUREMENTS OF UREA - 1-^{15}N BY CI-QUAD-MS
1000.0
100.0
10.0
1.0
PERCENT INCREASE IN RATIO OF m/e 62 m/e 61
0.01
0.1
1.0
10.0
PERCENT UREA -1-^{15}N

Fig. 2


ISOTOPE DILUTION MEASUREMENTS OF CHENODEOXYCHOLIC ACID -24-13C BY CI-QUAD-MS
1000.0
100.0
10.0
1.0
PERCENT INCREASE IN RATIO OF m/e 372 : m/e 371
0.1
1.0
10.0
100.0
PERCENT CHENODEOXYCHOLIC ACID-24-13C

ISOTOPE RATIO MEASUREMENTS BY CHEMICAL IONIZATION QUADRUPOLE MASS SPECTROMETRY

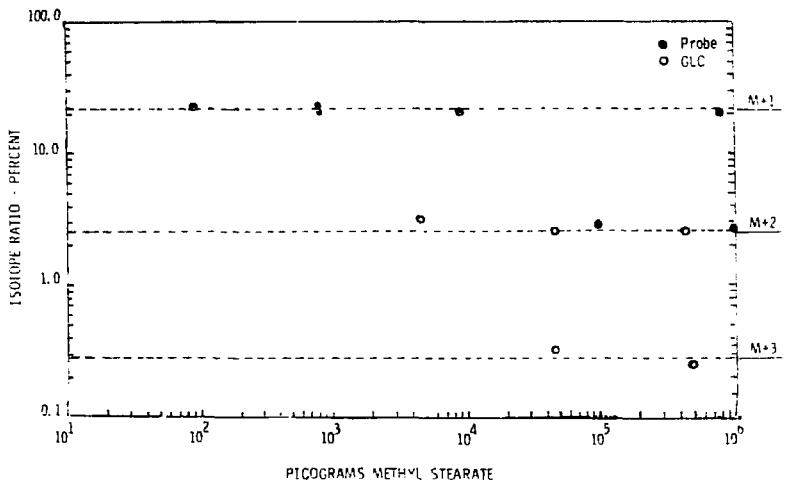
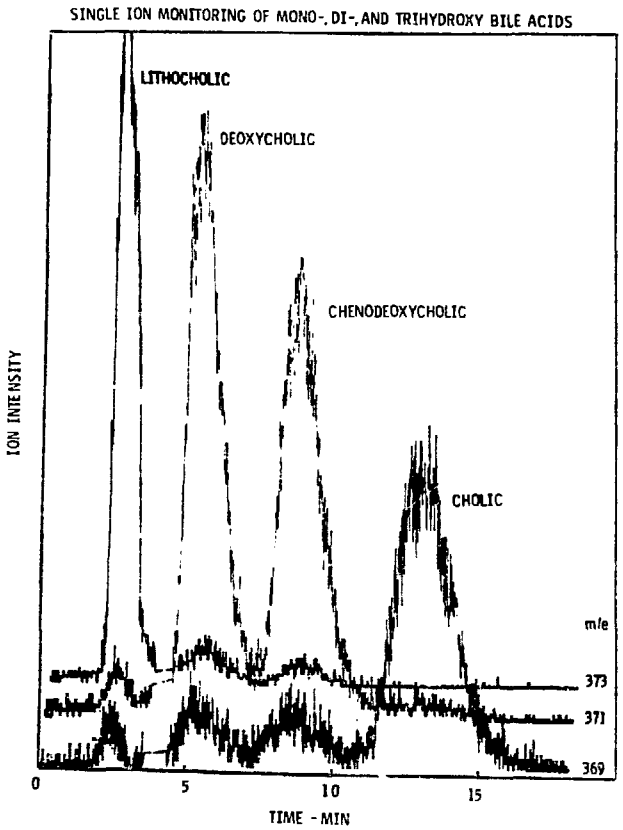


Fig. 4


SINGLE ION MONITORING OF MONO-, DI-, AND TRIHYDROXY BILE ACIDS
LITHOCHOLIC
DEOXYCHOLIC
CHENODEOXYCHOLIC
CHOLIC
ION INTENSITY
m/e
373
371
369
0
5
10
15
TIME - MIN