Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 6, pp. 735–745, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00

Bioanalytical mass spectrometry as applied to organic acid profiling and steroid reference methodology*

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Abstract: Gas chromatography–mass spectrometry (GC–MS) is a versatile and powerful diagnostic tool in clinical chemistry. Instrumental aspects of the technique are considered. The use of GC–MS for identification purposes is exemplified by the metabolic profiling of organic acids for the diagnosis of inborn errors of metabolism. Quantitative GC–MS is discussed with particular emphasis on the choice of the internal standard. For highest precision and accuracy, isotope dilution GC–MS (ID–GC–MS) is the most suitable technique; the application of ID–GC–MS to reference methods and reference materials is discussed in relation to the analysis of steroid hormones. The principles involved in devising calibration procedures and measurement protocols in quantitative GC–MS are outlined.

Keywords: *Gas chromatography–mass spectrometry (GC–MS); metabolic profiling; steroid reference methodology; isotope dilution GC–MS; calibration procedures.*

Introduction

Mass spectrometry (MS) continues to gain importance as an analytical tool to solve complex problems in biochemistry and medicine. For fundamental research in molecular biology and biochemistry, the focus now is mainly on very sophisticated MS techniques such as fast atom bombardment (FAB) mass spectrometry, laser microprobe mass analysis (LAMMA), thermospray LC–MS and MS–MS. In clinical chemistry, integrated gas chromatograpy–mass spectrometry (GC–MS) has proved to be a versatile and powerful diagnostic tool. Because of its unique capability for the separation and simultaneous identification of endogenous and exogenous substances with a molecular weight of up to 1000, GC–MS is especially suitable for multicomponent analysis, drug metabolism studies and peak recognition in, for example, a metabolic profile. The mass spectrometer can also be used as a very sensitive GC detector with tuneable selectivity and sensitivity. Typical applications in this field include the detection and determination of drugs, neurotransmitters, prostaglandins, steroids and trace elements; in addition

*Presented at the "Second International Symposium on Drug Analysis", May 1986, Brussels, Belgium.
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Table 1
Bioanalytical applications of GC–MS

Multi-component analysis of endogenous compounds: steroid hormones, bile acids, fatty acids
Metabolic profiling of organic acids and steroids
Identification of volatile constituents in lung, brain and liver tissues
Structure elucidation of drug metabolites
Tracing and identification of noxious compounds: drugs of abuse, industrial poisons, pesticides
Detection and quantification of trace elements, neurotransmitters, prostaglandins, steroids, etc.
Bioavailability and metabolism studies
ID–MS as a reference method

isotope dilution mass spectrometry (ID–MS) is used as a reference or definitive method (Table 1).

Instrumental Considerations

Packed analytical columns which, because of their well established characteristics, high sample capacity and instrumental design, persisted a long time in GC–MS work, have now largely been superseded by fused silica capillary columns. Their main advantages are a much higher separation efficiency, shorter analysis time and a better sensitivity because of the narrower band width of the eluting analyte, which largely compensates for the lower sample capacity. Moreover, since a single column can be used for a wide range of applications, the need for a frequent change of columns is eliminated and thus instrument down-time is reduced.

The introduction of the sample remains one of the most critical aspects in capillary GC. A detailed discussion can be found elsewhere [1]. For high precision quantitative work the authors recommend the use of an all-glass moving needle injection system [2–4] or a cold-on column injector, whereas a splitless injection system is preferred for multi-purpose use because of its versatility.

In modern instruments, the pumping capacity of the diffusion or turbomolecular pumps is usually sufficient to allow narrow-bore capillary columns to be coupled directly to the ion source of the mass spectrometer without the need for a molecular separator. In the authors' experience such a direct connection is highly recommended in terms of sensitivity (quantitative sample transfer) and inertness (no active sites at which biomolecules can decompose).

Many instruments today offer a choice between electron-impact (EI) and chemical ionisation (CI) methods, a choice which is particularly useful when one has to identify unknown compounds or when an extensive fragmentation in the EI mode reduces sensitivity [1, 5]. All the work described in the present paper, however, has been carried out in the more common EI mode.

The application of capillary columns requires the use of fast scanning devices (quadrupole mass analyser or laminated magnet) and improved data systems capable of

collecting enough data points across the narrow chromatographic peaks in order to maintain the required precision for quantitation. The data acquisition and handling in modern instruments is controlled by a mini- or microcomputer with a disk-oriented operating system, which also allows automatic calibration of the instrument, real-time graphic display of data and automated, mass spectral, library searching as well as several programs for data reduction.

Use of GC–MS for Identification Purposes

In the electron impact (EI) ionisation mode, positive ions are formed by bombarding the sample molecules with electrons. Upon collision, these electrons remove an electron from one of the outer molecular orbitals thus leading to the formation of a molecular ion M^+. Since the kinetic energy of the bombarding electrons (±70 eV) usually exceeds the ionisation potential of the molecules, the excess of energy absorbed leads to a number of fragmentation and rearrangement processes, which are more or less characteristic for the type of molecule or derivative. Thus the molecular ion yields information on the molecular weight of the compound whereas the fragment ions provide structural information. In the repetitive scan mode, the mass analyser is scanned continuously over a range of several hundred mass units in such way that with short intervals, e.g. every 2 s, a full spectrum is taken of the GC peak eluting at that moment (Fig. 1). All spectra are stored on disk and may be called up later for manual interpretation or automatic peak identification.

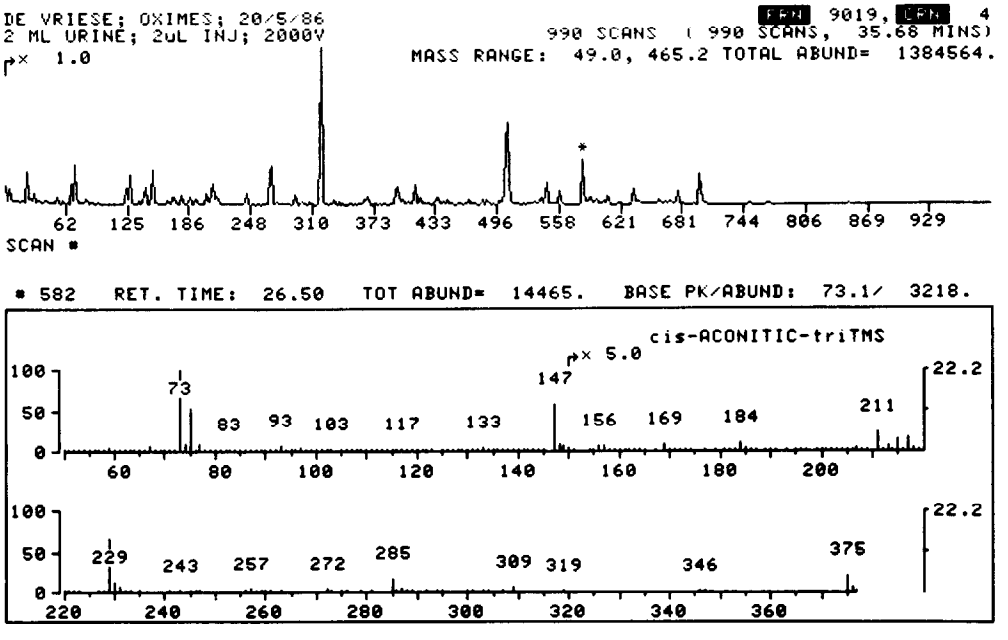


Figure 1
Upper trace: computer plot of the summed ion intensities (TI) of each scan versus the scan number, for a repetitively scanned GC run of trimethylsilyloxime derivatized urinary organic acids. Lower part: computer plot of the mass spectrum obtained at scan number 582, corresponding to the GC peak eluting with a retention time of 26.50 (*cis*-aconitic acid tris–TMS derivative).

Application to metabolic profiling

As a typical example of the use of GC–MS for identification purposes, the metabolic profiling of organic acids for the diagnosis of inborn errors of metabolism [6–8] will be discussed.

The primary cause of these diseases is a genetic defect in one of the genes which codes for enzymes involved in amino-acid, fatty acid, carbohydrate and/or intermediary metabolism. As a result, the affected enzyme is produced in inadequate amounts and the corresponding metabolic route is blocked. This leads to an accumulation of precursors, intermediates and abnormal side-products which are often toxic. If the patient is not treated in time, this in its turn may lead to very serious nerve and brain damage and result in severe mental and physical handicaps and/or death of the child. Thus an early diagnosis is required [9].

Many of these intermediates and side-products are organic acids and a gas chromatographic profile of the acidic fraction of urine will yield information on the presence and type of inborn error of metabolism. The profiles, however, often very complex, may vary from patient to patient and are further complicated by the fact that even very small peaks of highly abnormal compounds are diagnostically important (Fig. 2). Thus there is a clear need for a powerful identification technique such as GC–MS, especially if it is considered that some 60 different organo-acidurias are known involving a total of 200 different acid metabolites!

In the authors' laboratory the following approach [10] has been adopted.

Sample preparation. To a volume of urine corresponding to 1.5 mg of creatinine, 100 μg of 2-phenylbutyric acid is added as internal standard (IS). The urine is first treated with a solution of hydroxylamine hydrochloride at alkaline pH in order to convert the keto-acids into stable oxime-derivatives. This mixture is then acidified, salted out with NaCl and extracted twice with ethyl acetate. The combined extracts are evaporated to dryness and redissolved in 125 μl of *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA)–1% (v/v) of trimethylchlorosilane (TMCS) in dry pyridine (4:1, v/v) and allowed to react for 30 min at 60°C.

Gas chromatography with flame-ionisation detection (GC–FID) analysis. The resulting TMS–oxime derivatives are subsequently analysed on a combination of an apolar SE-52 and a semipolar OV-1701 capillary column with FID. By comparing the retention indices (methylene units) on both columns, a tentative identification of the peaks is made. Any sample representing an abnormality such as an increased concentration of a normal constituent, or abnormal metabolite pattern, is subsequently analysed by GC–MS on a HP 5985B mass spectrometer equipped with the same apolar SE-52 column as used for the GC–FID screening.

GC–MS analysis. After data acquisition in the repetitive scan mode, a total ion current (TIC) profile is produced and an automatic peak identification program is started. This compares the retention indices (methylene units) and mass spectra of the individual peaks with a reference library of some 200 different organic acid TMS and TMS–oxime derivatives. If both the retention index and the mass spectrum match with a probability ≥90%, the peak is identified and quantified by comparison of the ratio of peak area to that of the IS with data stored in a separate calibration file. For compounds which were not identified unambiguously, the spectrum is interpreted manually (see Scheme).

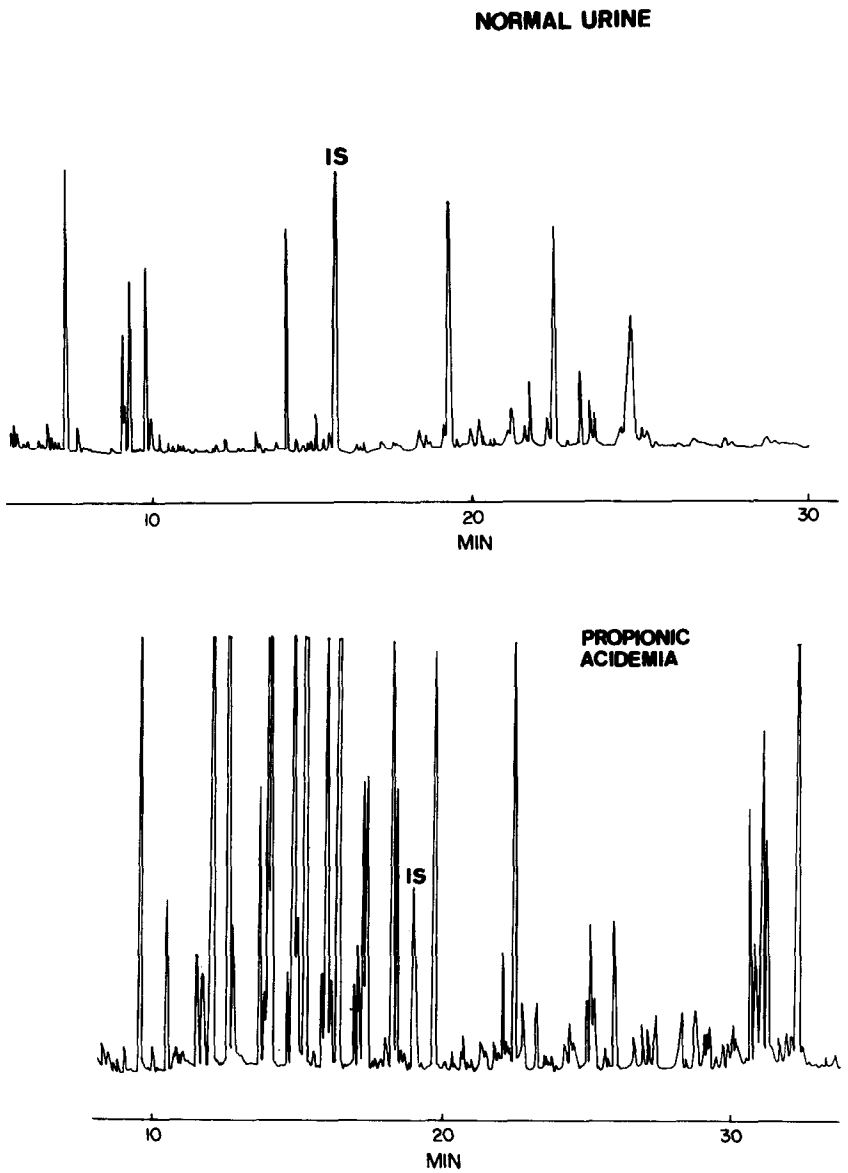
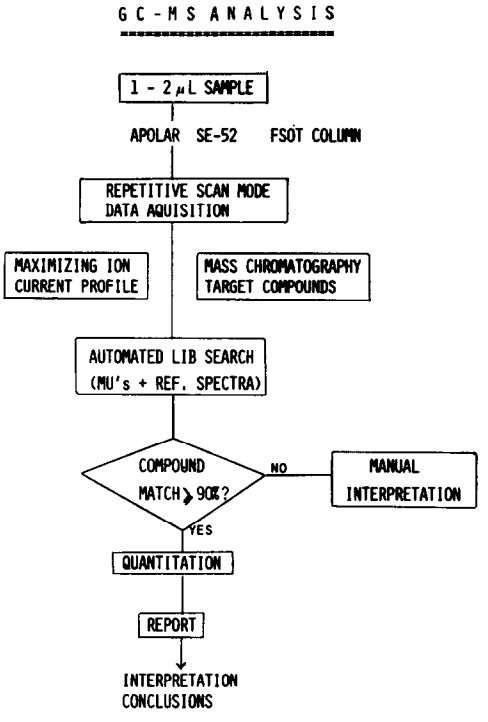


Figure 2
GC chromatograms of urinary acidic metabolites extracted from a normal urine (2A) and from the urine of a patient with propionic acidemia (2B). To both urine samples, the same amount of internal standard (IS) was added.

The raw mass spectral data obtained in the repetitive scan mode are stored on disk and thus are available for other data reduction programs such as a Biller–Biemann deconvolution or maximizing ion current profile analysis [11]. This program filters only those ions whose abundance increases in a particular scan. As a result, compounds whose retention indices are too close for resolution by chromatography but whose maxima show a minimal difference, can be determined separately. It should be noted however, that the



Scheme 1

corresponding mass spectra are the result of a mathematical data reduction and have to be interpreted with care. Thus, fragment ions which are common to both under-resolved peaks may be dropped. Mass chromatography on the other hand is used mainly to localize typical metabolites of a suspected metabolic disease. Here the computer is used to produce selected ion profiles of fragment ions typical for the compound(s) of interest (Fig. 3). After such a compound has been localized, however, its identity can still be further confirmed by recalling the complete mass spectrum from disk. The principal difference between mass chromatography and the technique of selected-ion monitoring (SIM) for detection (see next section) is indeed that the first is based on a computer reconstruction of full scan data whereas in the SIM mode the mass analyser itself is tuned to examine only a limited number of ions.

Quantitative GC–MS

Quantitative GC–MS has become at least as important in biomedical analysis as GC–MS is for identification purposes. With the availability of the newest generation of GC–MS instrumentation, which is designed for ease of operation and maintenance, it is possible to consider the MS just as a detector and quantitation device for GC. The basis for quantitative MS is the technique of "selected ion monitoring" (SIM) also called "mass fragmentography" (MF) [12, 13]. The key feature of this technique relies on the selective monitoring of only one or a restricted number of relevant m/z values of the analyte(s) of interest. Thus, the MS is functioning as a fixed mass filter which responds selectively to molecules which generate ions of the predetermined mass(es). Interference of other molecules with different m/z values is excluded. In coupling the MS to a GC, selectivity is

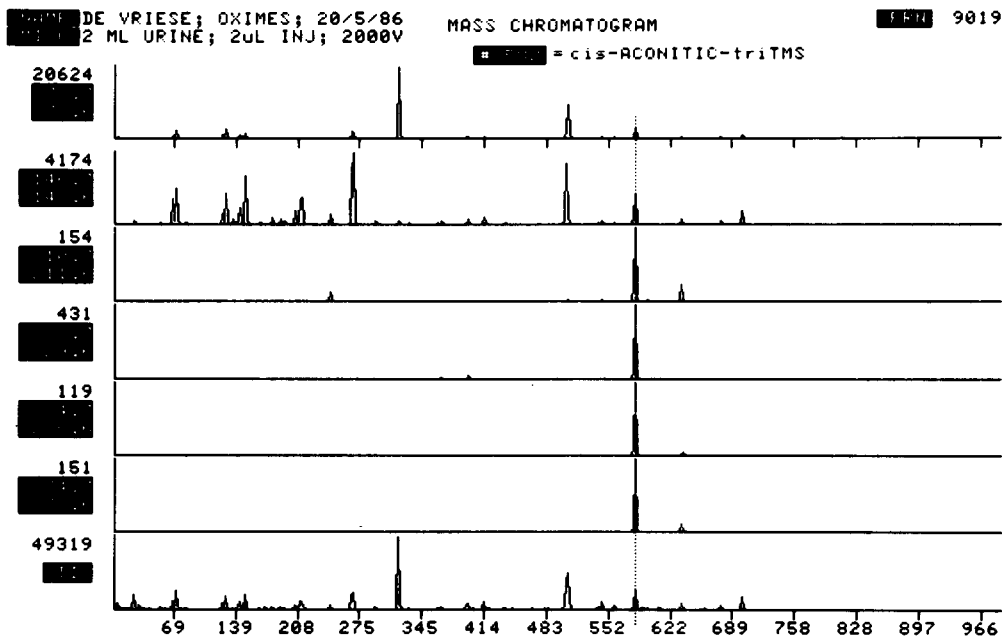


Figure 3
Computer-drawn ion intensity plots (mass chromatograms) of ions *m/z* 73, 147, 211, 229, 285, 375 and total ion intensity (TI) versus scan number from the same data file as used in Fig. 1.

further enhanced by the preliminary high-resolution capillary chromatographic separation of the different constituents present with the analyte(s) in the biological matrix. Because of the high degree of selectivity and the time averaging of the signal achievable in the fixed focus mode, the noise level in the MS signal is reduced and the sensitivity is increased by a factor of up to 1000 compared with that obtained for the same ions during a mass scan. This characteristic allows the measurement by GC–SIM of organic constituents in a biological matrix at the nanogram and even picogram level (Fig. 4).

Selection of an internal standard for quantitative GC–MS

One of the most important sources of error in analytical work in biochemistry lies in the sample work-up procedure. Therefore, all quantitative biomedical applications of GC–MS are based on the principle of internal standardization. In this technique, an IS is added at the earliest possible stage of sample manipulation to compensate for all possible losses through the whole analysis (extraction, purification, derivatization, etc.) until the final MS measurement. It is clear that only an "ideal" IS can fulfil these requirements; this makes the choice of IS one of the most crucial points in the design of a new assay. Theoretically, there are three possible choices; this was a subject of some controversy in the late 1970s [14–18].

1. A stable isotope-labelled analogue of the analyte, with the same retention time on GC but a different *m/z* value. This technique is called isotope dilution GC–MS (ID–GC–MS).
2. A close homologue of the compound of interest, which can be chosen to give the same *m/z* value but which has a different retention time on GC.

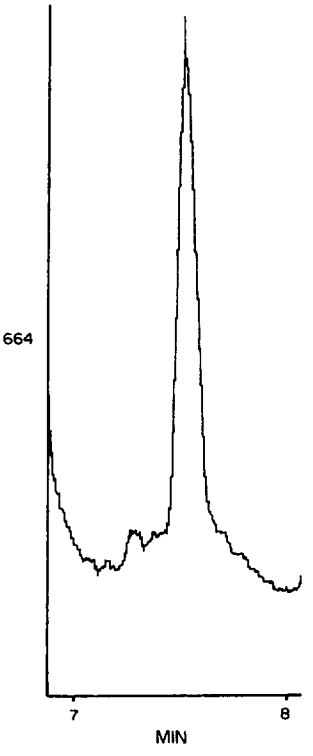


Figure 4
GC–SIM trace obtained from the extract of a serum sample containing 331 ± 6 pg ml^{-1} of 17β-oestradiol. The ion trace corresponds to the molecular ion (*m/z* = 664) of the di-heptafluorobutyric ester of 17β-oestradiol.

3. A compound of the same chemical class that may have either the same *m/z* value, in which case the retention time must be different, or a different *m/z* value, in which case the retention time may be different or the same. Since the first type of IS best resembles the analyte, ID–GC–MS is to be preferred whenever the objective is the highest obtainable precision and accuracy. As the number of ions produced during MS analysis is proportional to the number of molecules in the sample, quantitative determination can be done by comparing the ion abundance ratio of analyte in the unknown sample with that of standard mixtures with known amounts of analyte and the same amount of IS [1].

Application of ID–GC–MS to reference methods and reference materials

In the authors' laboratory, quantitative GC–MS is mainly applied to reference methods and materials. In clinical chemistry, the need for accuracy of test results is widely recognized [19–21] since it is directly linked to adequate patient care. A wide variety of techniques and methods for the quantitative analysis of biologically important parameters has been introduced. Until recently, the quality of these methods was mainly judged on the criteria of usefulness, reproducibility and cost without too much concern about accuracy. But it became clear in external quality assessment schemes that the use of so many different laboratory methods greatly and negatively influenced interlaboratory comparability and hence transferability of results [22, 23]. As a consequence, tendency has grown internationally to provide accurate analytical methods and materials to validate the accuracy of routine laboratory methods. The starting point to reach this

objective was the proposal and elaboration of a hierarchical structure of methods and materials [21, 24]. This structure includes definitive, reference and laboratory methods, in conjunction with primary, secondary reference and control materials. Each of the elements of the system is intended to guarantee transfer of accuracy from the highest level to the final routine level in the laboratory. It is envisaged that ID–GC–MS, which combines the separating power of GC with specific detection by MS and in addition allows the use of an "ideal" IS, provides definitive and reference methods for organic analytes [25–27].

ID–GC–MS for steroid hormone analysis

The GC–MS work on steroid hormones, performed in the authors' laboratory, is part of a project of the Bureau Committee of Reference (BCR), an EEC organisation that co-ordinates research groups in the validation and certification of human-based reference materials (RM). An expert group on steroid hormone analysis was asked to prepare and validate RM for cortisol (hydrocortisone), progesterone and 17β-oestradiol (E2) by ID–GC–MS. The appropriate isotope-labelled IS's are prepared by one of the members of the BCR expert group. On theoretical grounds, the molecular weight of the IS has to be increased, preferably by at least 3 amu, to avoid interference of natural isotopes of the analyte on the *m/z* value of the labelled compound [1]. In addition, the label has to be present at a stable position in the molecule and, of course, in that part of the molecule on which corresponding *m/z* value the MS is tuned. In practice, the BCR group used for cortisol, 1,2-$^2H_2$-cortisol; for progesterone and E2, 19-$^2H_3$-progesterone and 16,16,17-$^2H_3$-oestradiol, respectively. Details of the basic strategy for the development of reference methods in terms of the different steps in the analytical approach, can be found elsewhere; illustrations with a few selected examples from the authors' experience are included [1].

Calibration procedures and measurement protocols in quantitative GC–MS

Signal intensity in MS not only depends on the amount of sample but also on a number of MS variables: ionisation yield; focussing of the ion beam; and amplification by the detector. Despite rigorous control of MS parameters by microprocessors and dedicated computer software programs to reproduce the instrumental conditions of each run, instrumental drift can arise, i.e. ion abundance ratios can change with time. This effect can be minimized by the introduction of appropriate calibration procedures and measurement protocols. In this view, the NBS proposed the "bracketing" technique [28–30]. This involves the measurement of each unknown sample between measurements of calibration standards, whose ion abundances most closely surround the ion abundance ratio of the sample, in a total range of 15%. In addition, sample and standards are prepared in such a way that the ratio of analyte and IS is within a narrow range near 1:1. This scheme ensures the attainment of linearity and provides optimum measurement conditions [1].

Results and Discussion

Special attention has to be paid to the statistical treatment of the quantitative results. By means of an *F*-test the difference between the within-run and between-run variance is compared to evaluate the consistency of the measurement process. The results indicate whether significant differences exist between the means obtained during the different

Table 2
F-test on the results obtained by ID–GC–MS for 17β-oestradiol on the BCR candidate RM

Day	Mean (pg ml^{-1})	Number of samples	Standard deviation
1	331.0	6	5.67
2	332.4	4	7.94
3	330.9	4	6.32
SS_e: 6.05 (F = 2)		SS_i = 469.79 (F = 11)	
F_2, II = 11 SS_e/2 SS_i		p = 0.7	
Grand mean		= 331.38 pg ml^{-1}	
Total variance		= 36.60	
Relative standard deviation		1 = 1.83%	

days of analysis (Table 2). This test is decisive for the reliability of the grand mean or target value for the analyte in the RM.

Conclusion

Although the application of ID–MS as a reference or definitive method will always be restricted to a number of specialized laboratories, these two examples illustrate the great potential value of GC–MS as a diagnostic, highly accurate and precise analytical tool in clinical chemistry.

More detailed information on the current use and future trends of MS in clinical chemistry are given in a comprehensive review by Hill and Whelan [31].

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[*Received for review 4 August 1986*]