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Trends in biochemical and biomedical applications of mass spectrometry *

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ABSTRACT

This review attempts an in-depth evaluation of progress and achievements made since the last 11th International Mass Spectrometry Conference in the application of mass spectrometric techniques to biochemistry and biomedicine For this purpose, scientific contributions in this field at major international meetings have been monitored, together with an extensive appraisal of literature data covering the period from 1988 to 1991. A bibliometric evaluation of the MEDLINE database for this period provides a total of almost 4000 entries for mass spectrometry. This allows a detailed study of literature and geographical sources of the most frequent applications, of disciplines where mass spectrometry is most active and of types of sample and instrumentation most commonly used. In this regard major efforts according to number of publications (over 100 literature reports) are concentrated in countries like Canada, France, Germany, Italy, Japan, Sweden, UK and the USA. Also, most of the work using mass spectrometry in biochemistry and biomedicine is centred on studies on biotransformation, metabolism, pharmacology, pharmacokinetics and toxicology, which have been carried out on samples of blood, urine, plasma and tissue, by order of frequency of use. Human and animal studies appear to be evenly distributed in terms of the number of reports published in the literature in which the authors make use of experimental animals or describe work on human samples. Along these lines, special attention is given to the real usefulness of mass spectrometry (MS) technology in routine medical practice. Thus the review concentrates on evaluating the progress made in disease diagnosis and overall patient care As regards prevailing techniques, GC–MS continues to be the mainstay of the state of the art methods for multicomponent analysis, stable isotope tracer studies and metabolic profiling, while HPLC–MS and tandem MS are becoming increasingly important in biomedical research. However, despite the relatively large number of mass spectrometry reports in the biomedical sciences very few true routine applications are described, and recent technological innovations in instrumentation such as FAB-MS, electrospray, plasma or laser desorption have contributed relatively much more to structural biology, especially in biopolymer studies of macromolecules rather than to real life biomedical applications on patients and clinical problems.

INTRODUCTION

In the space allocated to this review an attempt is made to highlight the most significant contributions and trends in biochemical and biomedical mass

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TABLE 1

Accumulative number of documents related to mass spectrometry in the MEDLINE database [a]

Year	1988	1989	1990	1991
No of documents	1342	1235	1259	102

[a] Documents are classified by publication year from January 1988 to March 1991, inclusive. The revised June 1991 database was used for the search, with no documents appearing with a publication date after March 1991. A last-minute search, dated 16 July 1991, produced a total of 1338 and 314 entries for the years 1990 and 1991 respectively. This increase is due to late inclusion of publications from 1990 and early 1991.

spectrometry, as reflected in the current literature. For this purpose, the time span covered herein goes from 1988, the year of the last International Mass Spectrometry Conference in Bordeaux, to June 1991 or about the time of writing. The literature search is based on the MEDLINE database which provides almost 4000 entries in mass spectrometry (MS) within the period 1988 to March 1991 (Table 1). Such a broad literature base facilitates the overall evaluation of progress in this field. However, because of its large volume a comprehensive coverage is not feasible within the scope of this review. A fairly accurate representation of the present knowledge and status of biochemical and biomedical MS can be obtained from the proceedings of the specialized meetings that have been published within the last 3 years [1–4]. Recently, summarized reports of two of these meetings have appeared in the literature [5,6]. The pace of progress has certainly been impressive within the last 3 years, especially in the area of high mass measurement of biopolymers, until recently not readily amenable to mass spectrometric analysis. The new techniques now available for ionization of underivatized large, labile biological macromolecules, such as proteins, glycoproteins, glycoconjugates or oligosaccharides have virtually revolutionized the field of mass spectrometry to the point where nowadays we commonly refer to macromolecular mass spectrometry and the untapped research areas in structural biology that will benefit from these achievements.

Thus the excitement about the new techniques of mass spectrometry could accelerate the rate of sequencing in the Human Genome Project where MS isotope detection coupled to the ability to obtain molecular ion information of DNA molecules could lead to a replacement of electrophoresis in the analysis of Sanger-type sequencing mixtures [7].

In their superb review of mass spectrometry, Burlingame et al. [8] discuss and critically assess the evolution of biological mass spectrometry which has been especially remarkable in the area of new ionization techniques and instrumentation. In this area, it is worth mentioning that the rediscovery of classical atmospheric pressure ionization techniques [9] has certainly sim-

plified the interfacing of high performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), ion chromatography (IC) or capillary electrophoresis (CE) with mass spectrometry [10]. Likewise, time-of-flight MS (TOFMS), another technique known since the 1950s has seen a resurgence in the last years with the development of plasma desorption (PD) and laser desorption (LD) ionization of large biomolecules [11]. TOFMS is especially suited to macromolecular work owing to its unlimited mass range and provision of the complete mass spectrum per event. However, the most significant development for the near future may undoubtedly lie in the further development of the quadrupole ion trap [12]. The ion trap is notorious for its extremely high sensitivity (attomole range), its simplicity (the essential components would fit in one hand), relatively high mass range, resolution and compatibility with separation or ionization techniques.

Nevertheless, more than the significant advances in sophisticated and/or simple MS instrumentation the development and recent advances in matrix-assisted LD and electrospray (ES) ionization can be considered the most exciting events in MS within the last 3 years. Although the theoretical background supporting both ionization techniques dates from many years ago, both really took off in the MS field in 1988 and, especially for ES, have caught on very rapidly. For instance, while more than 30 papers on electrospray were presented at the 1990 ASMS meeting [13], this year's meeting in Nashville produced more than 80 papers on ES, many of them centred on real life applications and problem solving approaches rather than simply on development work. The simplicity of the technique which even lends itself to work with atmospheric pressure ionization (API) sources and the commercial availability of retrofit ES sources [13] undoubtedly has had a significant impact in the rapid implantation of ES ionization in mass spectrometric studies. The ES and related ion spray techniques are capable of producing a high yield of multiply charged ions, thus facilitating the analysis of biopolymers in standard quadrupole systems. In this regard, applications to the study of peptides and proteins in the 10–150 kDa mass range are already common at present and it has been shown that the mass range could be extended up to the 1–5 million mark providing the multiplicity of spectral peaks from such large ions can be resolved by existing mass spectrometers. An exciting possibility is the coupling of ES with the ion trap mass spectrometer, as recently demonstrated [14]. However, LD mainly produces singly charged ions with little or no fragmentation, thus requiring a TOF mass spectrometer to cover a very high mass range of up to 300 kDa [6], although it has also been recently interfaced to the ion trap mass spectrometer [15]. Both ES and LD MS have provided a major impetus in structural biology, allowing for instance the characterization of proteins or glycoproteins with a relatively high carbohydrate content [3]. However, as it will be discussed in some detail below, the techniques and

TABLE 2

Mass spectrometry in biological sciences. Number of reports per publication year (MEDLINE search)

	1988	1989	1990	1991 [a]
Behavioural and mental disorders	30	22	23	4
Biochemical phenomena, metabolism and nutrition	350	381	480	48
Biological phenomena, cell physiology and immunity	67	63	60	10
Biological sciences	7	17	13	0
Brain, brain chemistry	58	31	48	2
Circulatory, respiratory physiology	31	28	26	2
Chemical, pharmalogical phenomena	130	135	151	16
Digestive, oral, skin physiology	11	6	7	0
Environment and public health	126	118	151	10
Genetics	129	155	290	27
Health occupations	9	7	9	0
Musculoskeletal, neural, eye physiology	8	12	16	1
Physiology general	26	19	35	7
Reproduction, urogenital physiology	28	20	25	1
Toxicity, poisoning and intoxication	86	93	97	7

[a] To March 1991 inclusive.

instrumentation of mass spectrometry are clearly differentiated in their real life applications to biochemical or biomedical mass spectrometry and to macromolecular mass spectrometry, the latter setting the most modern and up-to-date trends in the field but the first being characterized by the use of more classical and well-established techniques.

BIBLIOMETRIC STUDY

As indicated above, a search of the MEDLINE database compiled at the US National Library of Medicine for the years 1988, 1989, 1990 and 1991 to March turned out a total of 1342, 1235, 1259 and 102 documents, respectively, for "spectrum analysis, mass" as the major subject heading (Table 1). This vast information has been classified in various topics and categories, enabling an analysis of trends in the application of mass spectrometry within the biomedical field. For instance, Table 2 illustrates the cumulative number of literature reports in different biosciences topics. As shown, this information indicates that most of the reports concentrate on biochemical phenomena, metabolism and nutrition followed by genetics, chemicals and pharmacologic phenomena, the environment and public health as well as toxicity, poisoning and intoxication. Also of some importance are the biological phenomena related to cell physiology and immunity as well as the neurosciences in general. An evaluation of this data points to relatively significant increases for the year 1990 in

TABLE 3

Medical subject headings (MEDLINE) chemicals and drugs

	1988	1989	1990	1991 [a]
Inorganics	293	237	316	30
Organics	619	584	628	48
Heterocyclics	300	268	268	18
Polycyclic hydrocarbons	158	154	143	13
Environmental pollutants	92	67	58	2
Hormones, antagonists	105	100	121	6
Reproductive control	10	17	15	1
Enzymes, coenzymes and inhibitors	219	199	277	38
Carbohydrates and hypoglycemics	194	183	194	22
Lipids and antilipemic agents	287	286	260	28
Growth substances, pigments, vitamins	56	59	63	7
Amino acids, peptides, proteins	337	303	381	31
Nucleosides, nucleotides	75	72	92	15
Central nervous system depresssants	108	78	133	7
Central nervous system agents	78	62	89	4
Autonomic drugs	84	63	91	4
Neuromuscular agents	6	6	9	0
Cardiovascular agents	58	40	58	2
Haematologic, gastric and renal agents	68	88	75	13
Anti-infective agents	81	97	87	9
Antiparasitic agents	8	11	14	0
Antineoplastics, immunosuppressants	45	42	44	5
Neuroregulator blockaders	29	32	30	1
Immunologic and biologic factors	206	185	176	28
Biomedical and dental materials	32	11	17	4
Miscellaneous drugs	333	238	287	23

[a] Number of MS reports in the MEDLINE database, classified by year of publication, to March 1991 inclusive.

the role of mass spectrometry in biochemical phenomena, metabolism and nutrition, and especially in genetics where the increase in the number of papers published in 1990 vs. the previous 2 years reaches 87%. Under the classification of genetics the database includes reports on genetic markers, metabolic disorders, inborn errors, genetic vectors etc. The distribution of applications according to the type of chemical or drug measured is shown in Table 3. Generically, organic and heterocyclic compounds are the most commonly studied although, surprisingly, there seems to be a good deal of work carried out also on inorganic compounds. It is evident that mass spectrometry plays a major role in the definition of the biochemistry and physiology of a vast array of biological compounds and xenobiotics influencing human health. Overall, the most striking trends in the figures in Table 2 are the increases in

TABLE 4

Medical subject headings (MEDLINE) diseases

	1988	1989	1990	1991
Bacterial and fungal	5	16	14	2
	(3,2)[a]	(9,7)	(8,6)	(1,1)
Neoplasms	32	43	35	4
	(12,23)	(26,25)	(25,15)	(3,3)
Digestive system	9	22	22	3
	(7,4)	(17,6)	(18,3)	(2,2)
Respiratory tract	7	8	13	2
	(5,2)	(6,3)	(10,2)	(2,1)
Nervous system	23	18	26	1
	(18,7)	(13,5)	(18,9)	(1,1)
Urologic and genital	14	14	15	3
	(11,4)	(12,5)	(12,4)	(1,2)
Cardiovascular	8	13	11	0
	(6,2)	(10,3)	(9,3)	(0,0)
Haemic and lymphatic	7	3	12	1
	(4,3)	(2,1)	(11,2)	(1,1)
Neonatal	5	7	6	0
	(4,1)	(7,0)	(6,0)	(0,0)
Skin	15	4	10	2
	(11,5)	(4,0)	(9,3)	(2,0)
Nutrition and metabolic	31	26	30	2
	(27,6)	(23,3)	(25,7)	(2,1)
Endocrine	15	16	20	1
	(8,8)	(13,3)	(16,7)	(1,0)
Immunologic	12	4	8	3
	(9,4)	(2,3)	(8,2)	(3,1)
Symptoms, general pathological	32	30	48	5
	(22,12)	(22,8)	(34,16)	(1,4)
Injury, occupational, poisoning	9	12	10	0
	(6,4)	(10,4)	(6,3)	(0,0)

[a] The numbers in parentheses indicate the number of reports describing the use of human vs. animal samples—left- vs. right-hand side numbers respectively. The sum of the two numbers does not necessarily add to the total indicated above because some manuscripts describe both humans and animal work and those are classified in both categories.

reports dealing with amino acids, peptides, enzymes, coenzyme inhibitors, perhaps reflecting the application of the new ionization techniques to biopolymer analysis and also central nervous system agents and depressants.

Likewise, the impact of MS on the study of the etiopathogenesis and course of various diseases is summarized in Table 4. There is a more or less even distribution of applications within the various disease states with the exception of the work related to neoplasms, the nervous system, nutrition and

TABLE 5

Distribution of reports found on the MEDLINE database on account of the type of sample used in the study

	1988	1989	1990	1991 [a]
Tissue	**109**/34 [a]	**123**/44	**91**/34	**11**/4
Blood	**248**/185	**223**/167	**225**/183	**14**/10
Plasma	**150**/94	**144**/102	**158**/99	**8**/4
Serum	**51**/44	**53**/42	**57**/45	**8**/5
Urine	**215**/154	**234**/158	**243**/181	**13**/9
Cerebrospinal fluid	**8**/6	**11**/8	**8**/6	**1**/1
Bronchoalveolar lavage	**3**/2	**2**/1	**4**/4	**1**/1

[a] To March 1991 inclusive.

[b] The bold numbers are for all reports referring to each sample type, irrespective of origin (human, animal, in vitro etc.). Numbers shown to the right of the slash are for human samples only.

metabolic diseases and general pathology where we can appreciate a relatively larger concentration of reports. This is not surprising except perhaps for the heading of neoplasms. A review of the pertinent literature references indicates that mass spectrometry has been instrumental in studies of antineoplastic and chemotherapeutic agents, their metabolism and citotoxicity, as well as in the characterization of antigens, inducible peptides, glycolipids, gangliosides cyclic peptides and mutagens in all kinds of carcinomas and neoplasm from brain tumours to colonic adenocarcinomas. All this work has been carried out in all kinds of biological samples, as shown in Table 5. As expected, the more restricted availability of human vs. animal tissue samples is clearly reflected in the much higher yearly ratios (all tissue samples/human tissue samples). However, the number of reports using the various types of samples is steady within the 3 year period, with blood and urine being those most frequently used.

Interestingly, the total number of applications of biochemical and biomedical MS in human and animal studies are evenly distributed as shown in Table 6, with a significantly reduced number of reports pertaining to work in vitro. The number of literature reports that make specific reference to case reports, patients, clinicial studies or work on inborn errors of metabolism is specified in Section I of this table. Also, the number of entries found in the database for work related in one way or another to diagnosis, disease, therapy, metabolism, biotransformation, pharmacokinetics is also specified in Section II (Total entries/human). As regards to the analytical procedures employed in all the entries in the database (Table 1) it is interesting to note that whereas at present the so-called biological or macromolecular mass spectrometry relies heavily on the new techniques for ion desorption and evaporation from liquid

TABLE 6

Number of entries for human vs. animal and in vitro studies

	1988	1989	1990	1991 [a]
I. Human	461	453	476	37
Case report	24	16	30	0
Patient	80	70	100	15
Clinical	33	34	39	9
Inborn errors	9	10	10	0
II. Animal	489	467	493	52
III. Total/human				
Diagnosis	**84**/59 [b]	**79**/46	**95**/41	**12**/9
Disease	**42**/30	**46**/32	**53**/24	**7**/4
Therapy	**30**/19	**27**/17	**48**/27	**9**/7
Metabolism	**527**/166	**518**/170	**556**/205	**66**/22
Biotransformation	**70**/17	**84**/31	**69**/21	**8**/2
Pharmacology	**143**/39	**145**/37	**172**/56	**20**/6
Pharmacokinetics	**93**/56	**83**/52	**103**/62	**9**/5
IV. In vitro	**72**/25	**58**/13	**55**/15	**6**/4

[a] To March 1991, inclusive.

[b] Bold numbers shown to the left of the slash are for all reports referring to each sample type, irrespective of origin (human, animal, in vitro etc.). Numbers shown to the right of the slash are for human samples only.

matrices as well as on the use of time of flight, standard tandem and sophisticated four-sector instruments, most of the work in the biomedical field is based on the more classical selected ion monitoring (SIM or mass fragmentography) techniques (Table 7). For determinations of high molecular weight compounds, fast atom bombardment (FAB) and secondary ion mass spectrometry (SIMS), especially the first, dominate the field while the applications of LC–MS as well PDMS and LDMS are still scarce despite the growing importance of these techniques in structural biology [3]. It is also of interest to note the parallel decrease since 1989 in publications based on thermospray (TS) and the rest of the LC–MS methods, which contrasts with the continued increase in ES LC–MS and the maintenance of the publication level in PD applications.

The total output from various countries for biochemical and biomedical mass spectrometry is shown in Table 8. Considering a minimum threshold of ten papers published within the 3 year period, a total of 21 countries can be represented in the table, although a few of them at a rather marginal level. It is evident that the major contributions, at least quantitatively, come from

TABLE 7

Techniques and instrumentation most often used in biomedical mass spectrometry

	1988	1989	1990	1991 [a]	Total
SIM	642	581	595	39	1857
SIM-CI	97	74	68	2	241
FAB	165	130	136	11	442
SIMS	23	28	22	1	74
MS-MS	58	61	64	4	187
LC-MS	17	39	16	1	73
TS	31	55	34	3	123
LC-FAB	1	3	2	0	6
ES	1	6	14	4	25
PD	16	16	14	3	49
LD	6	2	7	3	18

[a] To March 1991 inclusive.

TABLE 8

Distribution of reports in the MEDLINE database by country of origin

	1988	1989	1990	1991 [a]	Total
Australia	12	13	14	1	40
Austria	5	5	6	2	18
Belgium	20	15	9	0	44
Canada	39	47	53	4	143
Switzerland	17	11	9	1	38
Czechoslovakia	3	7	5	0	15
Germany	48	48	52	2	150
Denmark	6	14	9	1	30
Spain	15	3	6	1	25
Finland	12	11	15	0	38
France	62	54	51	5	172
Israel	9	4	6	1	20
Italy	33	49	23	3	108
Japan	134	123	145	13	415
Netherlands	28	28	23	2	81
Norway	11	6	3	1	21
Poland	5	3	4	1	13
China	6	2	2	1	11
Sweden	42	41	32	4	119
UK	68	45	60	1	174
USA	343	351	315	48	1057
Total	906	867	828	91	2692

[a] To March 1991 inclusive.

TABLE 9

Number of reports on human studies by country of origin (January 1988–June 1991)

	1988	1989	1990	1991	Total
Australia	6	4	5	1	15
Austria	3	5	4	0	12
Belgium	12	9	4	0	25
Canada	12	23	21	2	58
Switzerland	8	6	5	0	19
Germany	20	21	21	1	63
Denmark	1	6	3	0	10
Finland	6	4	5	0	15
France	20	23	21	1	65
Italy	15	18	10	1	44
Japan	50	38	60	5	153
Netherlands	11	10	15	1	37
Norway	6	2	2	1	11
Sweden	24	27	16	2	69
UK	28	22	39	1	90
USA	136	148	142	18	444
Total	358	366	373	34	1131

Canada, France, Germany, Italy, Japan, Sweden, the UK and the USA. However, owing to authors' imprecision in reporting their full affiliation with specific indication of country of origin, many of the documents in the database cannot be searched by country. For instance, if a publication shows Rome as the city of origin but Italy is not shown it will not be counted amongst the Italian entries. This is especially troublesome for the USA where most of the publications omit the country and show only the state or even in many cases only the city, like Bethesda. Presumably, all editors, referees and readers know where Bethesda is, but this certainly cannot be applied to places like Valhalla or Riverside. Thus the figures in Table 8 should be taken only as an approximation to the real numbers. The corresponding values for human only studies are indicated in Table 9. The number of countries is further reduced in this case since some of them did not reach the threshold value of a minimum of ten publications. However, the major contributors remain the same even to the point of maintaining their respective percentages relative to the total sum (Tables 8 and 9) except for Japan which goes down 1.9% and Sweden and the UK which increase by 1.6% their respective contribution in human studies by MS. All these contributions are distributed in over 300 journals, those shown in Table 10 being the most important in terms of the total number of publications they carry in biomedical MS. Again, the threshold value is set at a minimum of ten publications in total so that the

TABLE 10

Mass spectrometry in human studies. main publication sources

	1988	1989	1990	1991 [a]	Total
Anal Biochem	11	9	9	0	29
Anal. Chem	4	5	7	3	16
Arch Biochem Biophys	5	4	1	0	10
Arzneim.-Forsch.	9	6	4	0	19
Biochem. Biophys. Acta	4	10	4	3	21
Biochem Pharmacol	3	2	5	0	10
Biomed Chromatogr.	1	3	6	0	10
Biomed Environ Mass Spectrom	33	32	24	0	89
Clin Chem	19	16	10	2	47
Clin. Chim Acta	11	7	6	0	24
Drug Metab. Dispos	6	11	17	0	34
Environ J. Biochem	2	5	6	0	13
Forensic Sci. Int	4	4	13	0	21
J. Anal. Toxicol	20	17	20	0	57
J Biol Chem	16	20	18	4	58
J. Chromatogr	61	69	39	0	169
J Forensic Sci.	4	7	8	0	19
J. Inherit. Metab Dis	0	3	8	0	11
J. Lipid Res.	5	12	14	0	31
J Pharm Sci.	1	5	14	0	24
Methods Enzymol	4	1	11	0	16
Prostaglandins	6	5	5	0	16
Proc. Nat Acad. Sci. U S A	3	2	10	1	16
Steroids	1	1	12	0	14
Xenobiotica	7	5	7	1	20

[a] To March 1991 inclusive.

number of journals to be considered drops from over 300 to 25. As indicated in this table the journals concentrating a greater number of publications in this field are Analytical Biochemistry, Biological Mass Spectrometry (formerly Biomedical and Environmental Mass Spectrometry), Clinical Chemistry, Drug Metabolism and Disposition, Journal of Analytical Toxicology, Journal of Biological Chemistry, Journal of Chromatography and Journal of Lipid Research.

A consideration of the data contained in Tables 1–6 points to the futility of attempting to review all recent topics of interest on biomedical applications of mass spectrometry. Thus a practical decision was made to narrow the survey to studies carried out only on human subjects and human samples, whether in vivo or in vitro. Emphasis has been put on highlighting the real world connection between applications of state-of-the-art mass spectrometry and clinical medicine. As it becomes clear from the literature, the so-called

science of biomedical mass spectrometry has a much greater stronghold on biochemical and experimental research in general than in the truly patient-related clinical practice. In this regard and although work on experimental animal models as well as ex vivo and in vitro studies are of utmost importance in the development of reliable analytical methods for human studies, the restriction to MS literature describing work carried out on human samples and/or individuals leads to a more manageable situation. As illustrated in Table 6, the number of reports to be considered in this way is cut to less than one-half. For instance, as shown in Table 1 the MEDLINE database contains 1259 entries for 1990 related to mass spectrometry, in general. However, of these, 476 pertain to human studies and 493 to animal work with only 55 reports dedicated to in vitro studies (both human and animal). The rest of the 1259 or 235 reports correspond to other classifications such as studies describing the development of MS methods on authentic compounds.

Based on this selection of the database the following is a condensed account of the type of work reported from 1988 to and including March 1991 and classified according to a few major keywords, as indicated below. The major areas thus covered are grouped under the following headings: Exogenous Compounds: Drugs, Therapeutic Drug Monitoring, Pharmacology; Endogenous Compounds: Metabolism and Biochemical Phenomena; Diagnosis, Disease and Biochemical and Biological Markers; Isotopic Techniques; Case Reports and Clinical Studies; In Vitro Studies; High Mass Analyses.

EXOGENOUS COMPOUNDS: DRUGS, METABOLISM, THERAPEUTIC DRUG MONITORING AND PHARMACOLOGY

Mass Spectrometry has maintained a major role in the qualitative and quantitative determination of exogenous compounds ingested by humans either as pharmaceuticals, doping agents or street drugs. The number of reports dealing with these topics is relatively abundant and cover a wide range of compounds. In the area of pharmaceuticals and therapeutic drug monitoring a few examples could be cited, such as the pharmacokinetic studies on clembuterol in human plasma and urine at the femtomole level by negative ion chemical ionization GC–MS. Labelled clembuterol was used for the quantification and proper quality control and pharmacokinetic constants calculated from the MS data were reported. Furthermore, the authors claim a successful use of the technique in their laboratory over 4 years [16]. Two bronchodilators (Terbutaline and Orciprenaline) used in the treatment of asthma were studied by Leis et al. [17]. These authors also used negative ion chemical ionization (NICI) with isotope-labelled analogues for quantification of a highly electron capture sensitive derivative. On the other hand, Lindberg et al. [18] used positive CI to quantify bambuterol relative to a labelled analogue in plasma and urine. Bambuterol is a terbutaline prodrug which in the hands of these

investigators can be readily monitored in connection with pharmacokinetic and clinical studies. Quality control procedures were also properly implemented and the authors also claim successful use of the method for about 5 years.

Human pharmacokinetic data are also presented in a study of clebopride and its major N-*des*benzyl metabolite [19]. The method utilizes NICI with SIM and allows the detection of 0.1 ng ml^{-1} in human plasma. Likewise, the pharmacokinetics of aminoglutethimide was established by a SIM method, allowing the simultaneous detection of its N-acetyl metabolite as well as the study of single-dose kinetics [20]. Beclomethasone is used to treat patients with inflammatory bowel disease and its monopropionates and dipropionates have also been determined in human plasma and urine by LC–MS using NICI and a particle beam interface [21]. The thermal lability of synthetic corticosteroids can thus be circumvented by proper HPLC–MS techniques.

LC–MS has been rather extensively used for the analysis of pharmaceuticals and their metabolism. In this regard, mention can be made of its use to study the plasma clearance of anaethestic agents, such as Pancuronium and Vecuronium using a moving-belt HPLC–MS interface. In this case, thermal lability is used to dealkylate the quaternary ammonium steroids so that they go more readily into the gas phase. A deuterated internal standard was used and sufficient specificity was provided by B/E metastable transition monitoring [22]. Selected reaction monitoring (SRM) and API LC–MS–MS were also used in connection with the identification of the famous anabolic steroid stanozolol and its major metabolites in human urine [23]. Likewise, API LC–MS has allowed the determination of a renin inhibitor in human serum [24] using a heated nebulizer interface. This drug which is a modified tripeptide could not be analyzed by GC–MS owing to low volatility and thermal instability. The same compound could not be detected below 50 ng by thermospray, whereas the sensitivity of the heated nebulizer API source allowed a limit of quantification of 50 pg ml^{-1}. Thermospray (TS) however, has been successfully applied to the determination of pharmaceutical agents such as moricizine, a new antiarrythmic agent, and its labelled analogue in human plasma in a bioavailability study [25] or SK&F 101 468, a dopamine receptor agonist and its metabolites with a detection limit of 20 pg on a column [26]. According to the latter this is possible owing to the improved signal-to-noise ratio obtained using LC–MS–MS. In addition MS–MS provides structural information to compensate for the very scarce or total lack of fragmentation inherent to most of the TS mass spectra. However, as recently demonstrated, there are cases where TS can give sufficient structural information if minor but significant peaks are taken into account [27]. An example of the potential of continuous flow FAB is provided by Kokkonen et al. [28] in the analysis of erythromycin 2′-ethylsuccinate. The detection limit using the phase switching

system developed by these authors to enrich the analyte is only of 0.1 $\mu g\,ml^{-1}$ or 40 ng into the MS. The FAB MS–MS and TSPLC–MS on an EBQQ instrument were used for another antibiotic such as ampicillin and two of its metabolites [29].

Interestingly, there are a few reports dealing with practical applications of ion trap detection (ITD) systems to the determination of pharmaceuticals and their metabolites in human samples. For instance, beta blockers and their metabolites have been detected in urine, both in the full scan and MID mode of the ITD [30,31]. Also, an automatic reaction control CI technique in ITD was recently reported to quantify levels of arecoline, a cholinergic drug used in patients with Alzheimer's disease. Homoarecoline was used as internal standard and the CI reaction was carried out with acetonitrile instead of methane to avoid the significant hydride abstraction observed with the latter [32]. The Finnigan ITD 800 has been recently compared to another mass-selective detector (MSD) like the Hewlett Packard 5970 MSD, with similar results in terms of performance and sensitivity [33]. The MSD instrument has been used in automatic operation to quantify the metabolic profile of valproic acid [34]. The tetramethyl silane (TMS) derivatives were used to quantify 14 of its metabolites by GC–MS in the SIM mode. In a similar fashion Kassahun et al. [35] also identified 15 metabolites of the same anticonvulsant by NICI GC–MS of their pentafluorobenzoyl (PFB) derivatives. The MSD used in the scan mode also provided the identification of 15 metabolites of trimipramine, a tricyclic antidepressant [36].

Various applications of therapeutic drug monitoring and drug metabolic studies have been very recently reported in the Proceedings of the 2nd International Symposium on Applied Mass Spectrometry in the Health Sciences [4]. These include studies on the urinary metabolism of fenfluramine, [37], D.L. Kawain [38], coumarin anticoagulants by TSP and particicle beam (PB) LC–MS [39] and anabolic steroids [40] and diuretics by LC–MS [41], the latter two of interest in doping control. The screening of diuretics by GC–MS has also been reported recently by Lisi et al. [42]. In this case, an MSD system in the SIM mode was used. A study of the long-term use of single anabolic steroids and their metabolism by GC–MS indicates that their extended use resulted in a reduced excretion of urinary androgen metabolites but no changes in glucocorticoids [43]. Synthetic corticosteroid metabolism is of importance in controlling drugs of abuse in sport [44]. Biphosphonates in urine were analysed by GC–MS under EI, CI, NICI and CI–MS–MS conditions. These are synthetic compounds used for the treatment of hypercalcemia [45]. In this case the sensitivity of NICI–MS was superior to that of ammonia CI–MS–MS. On the other hand NICI–MS–MS was used to quantify indomethacin in 84 plasma and 84 synovial fluid samples of patients treated with the drug via topical application on the knee and found to offer a

sensitivity and selectivity high enough to allow the quantification of indomethacin down to 0.1 ng ml^{-1} using the deuterium-labelled analogue [46].

Drug metabolism is also an area where MS technique have been very actively pursued. For instance the metabolism of an oral dose of the anxiolytic agent buspirone labelled with $^{14}C/^{15}N$ was studied in human subjects. This work resulted in the unequivocal identification of seven metabolites and five other tentative structures presumably derived from the parent drug [47]. Along the same lines direct probe MS and GC–MS were used to identify metabolites of ethacizin, a cardiovascular active drug, and benzbromarone, used for the treatment of hyperuricaemia, respectively [48,49].

ENDOGENOUS COMPOUNDS: METABOLISM AND BIOCHEMICAL PHENOMENA

In a similar fashion as with the exogenous xenobiotics, mass spectrometry continues to be significant in studies on compounds and their metabolic pathways. The scope of compounds under study covers a wide range of biochemically active substances, both of low and of high molecular weight although the latter will be considered below in a separate section.

Prostaglandins E_2, E_1, $F_{2\alpha}$, $F_{1\alpha}$ and 6-keto $F_{1\alpha}$ in human and monkey cerebrospinal fluid have been measured by NICI of their methoxime-pentafluorobenzylester-trimethyl sylyl ether (MO–PFB–TMS) derivatives in the SIM mode. Although earlier literature reports had described concentrations ranging from undetectable to as high as 1 ng ml^{-1}, the authors did not find levels above the detection limit of 15 pg ml^{-1} [50]. Problems with earlier less-specific methodologies and especially with radioimmunoassay could be one explanation for the discrepancy. Immunoassay techniques were the basis of a method to isolate and purify a novel PGD_2 metabolite in human urine. The method allowed the recovery of 850 ng of the immunoreactive metabolite from 60 l of human urine. GC–high resolution SIM established the identity of the compound as 9-deoxy-δ-9, δ-12-dihydroPGD_2 [51]. Recently, Weber et al. [52] described a method for the quantification of 6-keto $PGF_{1\alpha}$, 2,3-dinor-6-keto-$PGF_{1\alpha}$, TXB_2, 2,3-dinor TXB_2, PGE_2, PGD_2 and $PGF_{2\alpha}$ in human urine samples using also NICI–SIM and with the same type of derivatives. Deuterated analogues were used as internal standards. The importance of being able to differentiate between 6-keto-$PGF_{1\alpha}$, TXB_2 and their respective dinor metabolites stems from the fact that the latter reflects global systematic production vs. local renal production for the first. In this regard, it has been indicated that 11-dehydro TXB_2, a major enzymatic metabolite of TXB_2, could be a more reliable index of endogenous thromboxane biosynthesis than TXB_2. Significant work along these lines has been carried out by Ishibashi and co-workers [53–55]. These authors have reported on the identification of 11-dehydro TXB_3 in human urine after administration of eicosapentaenoic

acid by high resolution SIM and more recently on a novel derivatization and immunoextraction method for 11-dehydro TXB_2 in human urine with detection by GC–MS–SIM of its methyl ester-11-*n*-propylamide-9,12,15-tris-dimethylisopropylsilyl ether. This derivative was used by the same group for the analysis of 2,3-dinor-6-keto $PGF_{1\alpha}$ [56]. Another method for 11-dehydro TXB_2 has been described by Lorenz et al. [57]. The metabolite was enriched by adsorption on phenylboronate cartridges and the corresponding Me-PFB ester quantified by isotope dilution NICI–MS–MS. The excretion of this compound was moderately elevated in heavy smokers and increased in patients with venous thrombosis or pulmonary embolism. Isotope dilution GC–MS has been used to quantify TXB_2, hydroxyheptadecatrienoic acid (HHT) and hydroxyeicosatetraenoic acids (HETEs) in human platelets [58].

Lipoxygenase metabolites of arachidonic acid have also been the object of much attention by MS procedures. For instance, 15-hydroxyeicosatetraenoic acid was detected by GC–MS–SIM as the major eicosanoid in nasal secretions [59] and Leukotriene B_4 was quantified in synovial fluid of osteoarthritic and rheumatoid arthritis patients by selected reaction monitoring GC NISI MS–MS [60] and in human serum [61], where it was shown that ex vivo formation can influence the assessment of circulating levels of LTB_4. More recently, the metabolism and elimination of Leukotriene E_4 has been reported by Sala et al. [62]. This work was elegantly carried out by infusion of radiolabelled isotopes of LTE_4 into male volunteers.

Another interesting observation made by mass spectrometry has been the discovery of long-chain saturated and unsaturated fatty acid carboxamides in plasma from drug-free women [63]. Along these lines, Harvey has reported on the identification of long-chain 12–26 carbon fatty acids and alcohols from human cerumen by use of esters which were capable of determining the position of unsaturation and methyl branching [64]. An ultrasensitive NICI GC–MS high resolution SIM method of phenylethylamine, an endogenous biogenic amine seemingly implicated in psychiatric illnesses, has been developed by Durden et al. [65] for its detection and quantification in 1 ml of human plasma.

NICI–MS has also been used for the determination of other neuroactive compounds such as Harman and *N*-τ-methylimidazoleacetic acid. In the case of Harman, this compound was detected in lung and human cerebrospinal fluid [66]. As it is a β-carboline it exerts a wide range of pharmacological activities and it has been reported to increase in brain following ethanol consumption. However, the MS data of Bosin et al. [66] suggests that Harman is unlikely to be formed as a result of ethanol ingestion so that its origin and pharmacological significance remain to be established. On the other hand, *N*-τ-methylimidazoleacetic acid is a major metabolite of histamine and thus of interest as an indicator of total histamine release. A method recently reported

for its determination in urine and plasma is based on SIM of the molecular ion of the corresponding isopropyl ester 3,5-bistrifluoromethylbenzoyl derivative and allows the detection of an amount equivalent to 1 pg of parent histamine [67]. Treatment of four mastocytosis patients with mast cell stabilizing drugs failed to alter the urinary excretion of these two compounds, as determined by the above method [68]. GC–MS in the SIM mode was used by Coldwell et al. [69] for the identification of vitamins D_2 and D_3 and seven of their 25-hydroxy,24,25-dihydroxy and 25,26-dihydroxy metabolites in a single 2 ml sample of plasma. According to the authors, this method demonstrates the potential of SIM as a possible reference method in this area. GC–MS has been also recently used by Harvey et al. [70] for the identification of urinary metabolites of cannabidiol in human urine. Another area where GC–MS has certainly continued being used with success is in steroid and bile acid analyses. Thus, very recently Fukushima et al. [71] have reported on the simultaneous determination of testosterone and androstadienone in human plasma by high resolution SIM using deuterium labelled standards in order to elucidate the in vivo conversion of testosterone into androstadienone. Likewise, a SIM stable-isotope dilution method has been reported for the determination of estrogens, androgens and progestins in follicular fluids aspirated from preovulatory follicles [72]. A total of five 16-androstenes with low olfactory thresholds were simultaneously quantified in extracts of men's axillary hair by GC–MS–SIM. The results indicated the existence of a pathway of metabolism in axillary bacteria in which 4,16-androstandien-3-one is reduced to 5α-androst-16-3-one and thence to 3α- and 3β-alcohols [73]. Five new sterols from fungi responsible for skin and nail infections were also identified by GC–MS–SIM [74]. Within the past 3 years, groups with an established background on bile acid analysis by mass spectrometry have continued publishing data of interest in this area. For instance Sjövall and co-workers have identified new C27 acids as normal constituents of plasma as well as neutral C27 steroids related to these acids, an observation which has led to the proposal of a model for the biosynthesis of bile acids under normal and pathological conditions [75–77]. An important finding, as claimed by these authors, is that levels of one intermediate in the biosynthetic pathway reflect the rates of bile acid biosynthesis. GC–MS and FABMS methods were used by the same group to identify novel glucosamine conjugates N-acetylglucosaminides excreted in human urine [78].

Another group active in this field has published reports on the study of bile acids in human foetal gall-bladder bile by a combination of techniques including FABMS for the direct analysis of bile acid conjugates and GC–MS to obtain a profile of individual bile acids after hydrolysis of the conjugate moiety [79]. The hydrolysis–GC–MS method was also applied to study bile acid metabolism in amniotic fluid with major changes being observed between early and late gestation in the human foetus [80]. The same group has

identified a new C4 hydroxylated bile acid in human foetal bile, accounting for 5–15% of total biliary bile acids in early gestation [81]. Plasma bile acids have been determined by isotope dilution capillary GC–NICI–MS down to the 1 pg detection limit [82]. GC–MS was also used by Hiraoka et al. [83] for the identification for the first time of bile alcohols in serum. A comparison of serum bile alcohol profiles indicated that the synthesis of urinary bile alcohols in healthy humans is of hepatic rather than renal origin. Keto and non-keto bile acids in human serum were determined by GC–MS–SIM [84] whereas Bjorkhem and co-workers used a specific SIM technique for the determination of unconjugated cholic acid in portal venous and systemic venous blood in healthy subjects, patients with ileal resection and patients with bacterial overgrowth of the upper small intestine, the results indicating that the proportion of unconjugated cholic acid in peripheral venous blood could be useful for the detection of bacterial contamination in the small intestine [85]. The same group has presented data on the products of cholesterol autoxidation in healthy subjects by an isotope dilution MS method [86].

Whereas most of the work in this area has been carried out by GC–MS or FABMS techniques, Setchell and Vestal described the advantages of thermospray ionization as a highly specific technique for bile acid analysis [87]. Combined HPLC–MS techniques have also contributed to other areas of endogenous compound metabolism such as in the identification of seven retinol metabolites, one of them for the first time [88] and quantification of endogenous tretinoin and isotretinoin, both of them retinoids, by HPLC–DLIMS in conjunction with normal phase liquid chromatography on microbore columns [89]. Thermospray isotope dilution HPLC–MS has been likewise used in the quantification of dehydroepiandrosterone sulphate in serum samples [90].

Low molecular weight endogenous peptides such as methionine enkephalin have also been the object of MS studies. For instance, Desiderio and co-workers have recently reported FABMS data on this peptide in human pituitary tissues. Highly specific measurements were possible by selected reaction monitoring of the ion current from a unique peptide fragment ion with the d5-peptide as internal standard [91,92]. β-Endorphin was also characterized and quantified in human pituitaries by FAB B/E linked-field scan MS–MS techniques [93]. In this case, since the peptide has a molecular weight of 3463 u the HPLC enriched fraction was treated with trypsin to produce shorter peptide fragments. More recently, the same authors have described the use of electrospray ionization for the determination of these two peptides, also in human pituitaries, thus achieving improved signal-to-noise ratios [94]. FABMS has also played a role in the structure elucidation of pentosidine. This compound, found in a variety of human tissues, is composed of an imidazo[4,5,6]pyridinium molecule comprising a lysine and an arginine residue cross-linked by a pentose. The cross-linking of the two amino acids is

postulated to have occurred as a consequence of a Maillard reaction with a pentose, a process that could be implicated in the aging process [95]. Standard GC–MS techniques have also been useful for the study of urinary nucleosides. Fractions obtained by HPLC were characterized by GC–MS of the corresponding TMS derivatives [96,97].

DIAGNOSIS, DISEASE, BIOCHEMICAL AND BIOLOGICAL MARKERS

Mass spectrometry, as indicated in Table 4, finds widespread use in the study of a wide array of human diseases, whether it is in the study of metabolic abnormalities leading or resulting from these diseases or as an aid in the diagnostic process. Diagnostic procedures on humans are usually tied up to non-invasive techniques. In this regard mass spectrometry provides a uniquely sensitive and highly selective means of readily assaying collectable physiological fluids such as blood, amniotic fluid or urine. Analytical methods based on mass spectrometric techniques can be tailored either to the detection or monitorization of one of a few related metabolites or to the large-scale screening for one or various families of compounds. In all cases what the clinician looks for are tell-tale signs of disease to help guide his diagnosis and therapy. These are the so-called biochemical markers of disease.

Large-scale screening procedures have been lately exemplified by reports such as that of Shoemaker and Elliott [98] on the automated screening of urine samples for carbohydrates, organic acids and amino acids. The GC–MS system set up by these authors is able to quantify 103 compounds in 6 min relative to endogenous urinary creatinine. The method relies on the removal of urea by the enzyme urease. This renders minor components in the sample accessible to the trimethylsilylation reagent used for sample derivatization. The diagnosis confirmed in this way on 104 urine samples provided by a paediatric hospital and other laboratories included various organic acid acidurias, ornithine transcarbamylase deficiency, lysinuric protein intolerance, glycinuria, and maple syrup urine disease.

A similar approach to the computerized screening of breath biomarkers in lung cancer has been described by O'Neill et al. [99]. This report is based on the classification of human expired air components into 16 chemical classes, based on empirical formulae, and the classification of components at the 75% and 90% occurrence levels. A total of 386 components were detected by GC–MS of which only 28 were present at the greater than 90% occurrence level. The quantitative GC–MS analysis of 61 organic acids, aldehydes and ketones in urine, plasma and amniotic fluid was reported by Hoffmann et al. [100]. As claimed by the authors the total organic acid content of the sample provides a rapid screening test for metabolic abnormality.

In terms of more specific compound searches, organic acid acidurias and

acidemias have attracted a lot of attention in the past since suitable biochemical markers of these conditions can readily determined by mass spectrometric techniques. For instance, 4-hydroxybutyric aciduria is an inborn error in the metabolism of 4-aminobutyric acid (GABA) which is due to a deficient activity of succinic semialdehyde dehydrogenase. The development of a stable-isotope dilution CI GC–MS of the TMS derivative of 4-hydroxybutyric acid allowed rapid prenatal diagnosis with a normal foetus being detected in a pregnancy at risk [101]. Also, GC–MS methods have been extensively used for the prenatal diagnosis of 76 pregnancies at risk in a 4 year time span [102]. This type of inborn errors of organic acid metabolism is important in that they have been shown to account for a proportion of sudden infant deaths. In medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, which is due to a defect in mitochondrial fatty acid oxidation the diagnosis becomes difficult because of the need to use cumbersome enzymatic assays. Thus, to prevent sudden deaths it would be convenient to be able to rely on a more convenient diagnostic method capable of identifying cases at an asymptotic stage. Along these lines Downing et al. [103] have published a GC–MS study on the urinary organic acid profile in the 5 days of life in 180 subjects. They diagnosed MCAD in a sibling of a sudden infant death case and concluded from their study that in neonates moderate generalized dicarboxylic aciduria is a harmless anomaly reflecting some immaturity of the fatty acid β-oxidation pathway and that in the neonatal period qualitative assessment of the overall pattern is more important than quantification of individual metabolites. Amongst the abnormal organic acids excreted in MCAD are the glycine conjugates of hexanoic, suberic and phenylpropionic which have been determined by ammonia CI–SIM in 54 urine samples of 21 MCAD-deficient patients [104]. As claimed by the authors these acylglycines, although detected earlier, lacked a clear diagnostic value owing to inappropriate analytical methods.

A further diagnostic aid in the recognition of organic acidurias seems to rely on the quantification of carnitine and acylcarnitine profiles which have been extensively investigated by Millington and co-workers [105] mainly by FABMS–MS [106,107] and continuous flow FAB–MS [108]. These authors have shown a simple way to distinguish acylcarnitines from interferences in a biological matrix by performing the MS analysis after an oral ingestion or intravenous infusion of stable-isotope-labelled l-camitine. Very recently Millington et al. presented an account of the biomedical applications of continuous flow FAB–MS [109] which includes compound specific detection of diagnostic acylcarnitines in human urine. Carnitine is known to play a role in fatty acid oxidation and their mitochondrial transport. Thus there is interest in establishing the effect of intravenous L-carnitine on the metabolism of fatty acids, ketone bodies, glucose and branched chain amino acids. In this regard, GC–MS has been used to determine a lack of effect on the intermediary

metabolism which casts doubt on the role of carnitine in the treatment of various medical conditions [110].

Some other acids of diagnostic interest are, for example, methylmalonic (MMA), hippuric (HA) and 3-carboxy-4-methyl-5-propyl-2-furanpropionic (FA) acids. In the case of MMA a simple GC–MS–SIM stable isotope dilution method has been reported for its determination in serum [111]. The method is useful for the diagnosis and evaluation of cobalamin deficiency. As for HA and FA, both are important for the study of renal excretion function in kidney diseases since they accumulate in renal failures. These acids were determined in serum, plasma and urine by GC–MS [112]. Plasma levels of both acids in haemodialysed patients with chronic renal failure were elevated in comparison with controls or patients without renal disease. Other authors have studied by GC–MS the in vivo oxidation of infused (1-^{13}C)-propionate and urinary metabolite excretion in children with methylmalonic acidemia, with the conclusion that this is an important route of propionate disposal [113].

The mass spectrometric determination of abnormal fatty acid metabolism in hopantenate therapy during clinical episodes has been reported recently [114,115]. The presence of very long chain fatty acids in plasma is an important diagnostic criterion for the diagnosis of peroxisonal disorders as in adrenoleucodystrophy, Zellweger and infantile Refsum's syndromes. These compounds have also been determined by GC–MS techniques [116,117].

Cholesterol metabolites can be also useful as biomarkers of disease states. Along these lines, increased urinary excretion of bile alcohols in cases of primary biliary cirrhosis [118] and liver dysfunction [119] has been demonstrated by GC–MS techniques. The GC–MS and GC–MS–SIM procedures have also provided evidence for the presence of unusual bile acids in liver diseases [120–122]. This has certainly been a very active field which has attracted a great deal of attention from various groups reporting applications of GC–MS techniques to the study of bile acid metabolism in cerebrotendinous xanthomatosis [123], liver diseases [124], peroxisomal thiolase deficiency [125] and cystic fibrosis plus associated liver disease [126] before and after ursodeoxycholic acid administration. In this case FABMS was used besides GC–MS to identify more than 50 individual bile acids, mainly as their glycine and taurine conjugates. GC–NICI–MS and GC–MS methods were developed for the determination of 5β-cholestanoic acids in human urine and they were applied to the separation and quantification of these acids in patients with the cerebro–hepato–renal Zelleweger syndrome, a rare hereditary disease characterized by a lack of peroxisomes in hepatocytes [127–129]. Another group very active in this field is that of Sjövall and co-workers who have continued to produce a fair amount of significant work in the biosynthesis of bile acid markers of disease. Thus levels of newly identified C27 bile acids in plasma [130] were affected by diseases of the liver (primary biliary and

alcoholic liver cirrhosis) [131] and the distal ileum [132]. All of this work was carried out by GC–MS procedures which were also used to demonstrate that bile alcohols inhibited 7α-dehydroxylation by intestinal bacteria. This would explain why deoxycholic acid is not formed in patients with cerebrotendinous xanthomatosis [123], a familial disease characterized by deposition of cholestanol and cholesterol in brain and tendons [133]. This group also used FABMS and GC–MS methods for the study of a new inborn error of bile acid biosynthesis. A child case history and the bile acids excreted by this child indicated a lack of 3β-hydroxy-δ-C27 steroid dehydrogenase/isomerase. With these data in hand, an appropriate bile acid treatment could be introduced to achieve effectively the recovery of the child [134]. Using FABMS screening procedures to study urine extracts from other children with liver disease, additional defects in bile acids biosynthesis were established. For instance, a reduced activity of hepatic 3-oxo-δ-4-steroid-5β-reductase was determined in children with severe liver disease [135]. Although the causes of these metabolic defects are not clear at present, Setchell et al. [136] have evidence for an inherited deficiency of 5β-reductase in a pair of twins with neonatal hepatitis. Furthermore, 3β-hydroxy-δ-5-C27 steroid dehydrogenase deficiency has now been detected in two additional patients [137]. Some special ketonic bile acids have been shown to be excreted by newborn infants [138] and these disappear with age [139], except in the presence of liver disease [140]. In the area of defective cholesterol metabolism, Shackleton and Reid have reported a quantitative isotope dilution HPLC–thermospray MS method for cholesterol sulphate in plasma which could be of interest in the diagnosis of recessive x-linked ichthyosis [141]. According to these authors, although the instrumentation required is complex the assay is simple and they have diagnosed 24 patients.

Another isotope dilution MS method for cholestanol sulphate in plasma has been recently reported by Veares et al. [142]. In this case the compound was quantified by FABMS which requires the use of a stable isotopically labelled analogue to compensate for the wide and unpredictable variability of FAB responses. A comparison of the FAB data and GC–MS–SIM assays gave superimposable calibration curves confirming the absence of any systematic error in the analytical protocol. A similar approach was used by Lund et al. [143] to determine serum levels of unesterified lanosterol.

Other compounds such as eicosanoids [144] or quinolinic acid [145] also offer potential as diagnostic aids. In the case of quinolinic acid the correlation established between the level of this exocitotoxic acid and clinical and neurological status makes it a predictive measure of infection status such as clinically overt AIDS dementia complex, aseptic meningitis, opportunistic infections and neoplasms. An impressively low limit of detection of 0.4 fmol or 67 fg has recently been reported for a GC–MS determination of its dihexa-

fluoroisopropyl ester [146]. Urine glyceraldehyde excretion has been measured by GC–MS in children with the renal Fanconi syndrome and in normal individuals. Apparently, results show that marked excretion is a previously unrecognized feature of this syndrome [147]. Finally, phenylalanine is another compound of diagnostic interest for which a candidate reference isotope dilution MS method has been recently proposed [148].

ISOTOPIC TECHNIQUES

The remarkable specificity and sensitivity of mass spectrometric techniques makes them the methods of choice for the detailed study of the metabolism and kinetics of both endogenous and exogenous compounds. This type of approach involves the administration of the isotopically labelled compounds to human volunteers and the detection and identification of labelled biotransformation end-products. The use of stable isotopes, however, presents some clear advantages over radioisotopes in that health risks related to radiation exposure are prevented, and MS can identify the site of labelling as well as the identity of an isolated metabolite. At the same time these are much more accurate and technically simpler to perform that the labour-intensive processes of chemical degradations needed to characterize isotopic distributions. Along these lines, several of the reports that have appeared within the last 3 years are worth mentioning.

For example, deuterated acetanilide was used as a probe to study the metabolism of acetanilide in man and the rat [149]. In this study the extent of deacetylation–reacetylation is taken as an indication that deacetylation pathways could modulate the distribution of acetylator phenotypes.

A similar GC–MS approach was used more recently for the simultaneous determination of nicotine, nicotine-3′,3′-d_2 and the metabolites cotinine and cotinine-4′,-4′-d_2 in human plasma after the infusion of a 50:50 mixture of nicotine and nicotine-d_2. This method is claimed to be useful for quantitative studies of nicotine metabolism [150]. Selected ion monitoring over a limited mass range has been used for the rapid measurement of whole-body and forearm protein turnover using a primed constant infusion of $^2H_5$ phenylalanine [151]. The method obviates the need for measurement of expired air CO_2 production and ^{13}C enrichment by isotope ratio mass spectrometry (see below) as required in the more standard 1-^{13}C leucine technique. Continuous infusions of sodium $^2H_2$- and ^{13}C-propionate were used to determine propionate turnover by CI–MS–SIM [152]. The same MS technique was also used to determine ^{15}N enrichment in studies on the bioavailability of dietary urea nitrogen in the breast-fed infant [153]. Stable isotope infusion GC–MS studies on steroid metabolism [154] and kinetics in pregnant women have shown that sulphated 3-hydroxy-5α steroids may account for 50% of the

metabolism of progesterone in late pregnancy [155]. Also, in the area of steroid metabolism it has been acknowledged that radiotracer methods for the study of cortisol production rates in physiologic and pathologic states in humans have provided conflicting evidence. However, a recent report described the first method for the direct determination of 24 h plasma cortisol production rate during continuous administration of a stable isotope by thermospray LC–MS, which avoids the use of derivatization procedures allowing on-line detection of plasma cortisol after a simple extraction procedure [156].

Mass spectrometry in the SIM mode of detection also facilitates the study of glucose turnover. For instance, even though hepatic glucose production and glucose carbon recycling are usually evaluated by the combined use of hydrogen and carbon-labelled glucose tracers, single-isotope methods require the determination of activities in different glucose carbon atoms by chemical degradation procedures. Also (U-^{13}C6)-glucose is still too expensive for routine analysis whereas the ^{13}C content in the different positions of the glucose carbon skeleton can be readily determined by SIM. Along these lines, a recent report describes the use of (1-^{13}C6)-glucose tracer for the measurement of recycling by SIM. This provides a single-label, non-radioactive method for the determination of hepatic glucose rates [157]. Almost concurrently another group has described the use of uniformly labelled ^{13}C-glucose for the determination of glucose turnover using a mass-selective GC–MS–SIM system [158]. Other authors have established also by GC–MS–SIM techniques that measurement of glucose turnover with (6-^{3}H)- and (6,6-$^{2}H_2$)-glucose leads to an underestimation of the true values during insulin infusion studies and that this was not the case with (6-^{14}C)-glucose during hyperinsulinemia [159]. However, MS measures of isotopic enrichment of (6,6-$^{2}H_2$)- and (2-^{2}H)-glucose tracer in the intravenous glucose tolerance test (IVGTT) suggested that during an IVGTT there are minor differences in basal insulin-derived measures because of metabolic differences in the fates of the tracers and that these are rather small for parameters describing insulin-stimulated processes [160].

A related technique which has played a significant role in human metabolic studies is isotope ratio mass spectrometry (IRMS). Continuous flow IRMS was previously used for the measurement of ^{13}C and ^{15}N in biological tracer and clinical substrate metabolic studies and more recently a novel continuous flow IRMS method was described for the analysis of ^{18}O enrichment in urine. The method was applied to studies of total body-water and water turnover in the clinical field [161]. IRMS has also been used recently for the measurement of short-term triglyceride synthesis in four healthy males by determining the incorporation rate of deuterium in body water into plasma triglycerides [162]. The authors claimed that the technique could be useful in studying the kinetics

of lipid disorders and dietary responsiveness of triglyceride production. Another recent and interesting application of IRMS lies in the measurement of ^{13}C-arginine incorporation into apolipoprotein B-100 in low density and very low density lipoproteins in normal subjects after an infusion of ^{13}C sodium bicarbonate [163]. The method is safe for use in children and applicable to a wide range of plasma proteins facilitating the studies of alterations and kinetics of Apo B biosynthesis in disorders such as the genetic hyperlipidemias. Finally, IRMS using a thermal ion source was used in a study of the kinetics of intestinal calcium absorption using oral ^{44}Ca and intravenous ^{42}Ca isotopically enriched stable calcium tracers [164]. Other elements which have also been the object of MS work are iron and zinc. In this case the absorption of the stable isotopes ^{58}Fe and ^{67}Zn in human subjects fed a mixed meal of extruded and non-extruded wheat bran and flour was measured from foetal excretion samples by FAB [165].

As indicated above both GC–MS in the SIM mode and IRMS have been very useful for measuring isotopic enrichment rates, the latter being two orders of magnitude more precise but also more costly. In this regard thermospray LC–MS could become a useful alternative as recently demonstrated for the first time [166]. As claimed, the advantages would include speed of analysis, ease of sample preparation, sensitivity, reproducibility and a chromatographic separation system that obviates the need for sample derivatization. The use of stable isotopes can also provide an alternative to biopsy as shown in a study where the natural ^{13}C enrichment of leucine in plasma and muscle protein was measured and compared [167]. The results indicated that ^{13}C enrichment of leucine in plasma protein reflects that of muscle protein and provides an attractive alternative to additional muscle biopsy. Furthermore, an NICIMS method for the isotopic determination of organic α-keto acids in human plasma has been recently reported [168]. The method allowed the studies of isotopic enrichment in 4-methyl-2-oxopentanoic, lactic and pyruvic acids and leucine in volunteers and subjects with maple syrup urine disease who had received infusions of (^{13}C)-alanine and ($^{2}H_3$)-leucine. GC–MS of the 3,5-dinitrobenzoate derivatives of deuterium-labelled ethanol infused in vivo into healthy volunteers has allowed the study of the mechanism of ethanol elimination in humans [169].

CASE REPORTS AND CLINICAL STUDIES

Although the biochemical characterization of clinical conditions through metabolic studies of specific disease biomarkers has already been described above, a further consideration is given in this section to actual reports highlighting the utility of mass spectrometry in clinical situations. Furthermore, since lack of space precludes a detailed consideration of the role of MS in

studies related to toxicology and poisoning, this section will include a few case reports arising from human accidental intoxication or suicide attempts. For instance, in a case where the patient's clinical presentation and laboratory findings were indicative of acidosis a GC–MS analysis of serum specimens revealed that the acidosis was caused by ketone bodies and a high concentration metabolite of toluene, benzoic acid. This case was referred in the literature as an unusual case of toluene-induced metabolic acidosis [170]. Mass spectrometry was also involved in the diagnosis and study of a new case of D-glyceric acidemia/aciduria in a 9-month-old girl, the fifth of such reported cases. This was discovered during GC–MS screening of urinary organic acids [171]. A fluoxetine overdose leading to the death of a 28-year-old white female was confirmed by GC–MS identification of fluoxetine and its metabolite, norfluoxetine [172]. The GC–MS methods have played a major role also in other cases of human intoxications and fatalities due to drug overdosing by compounds such as fentanyl [173], hydroxychloroquine [174], cyclicine [175], methomyl [176], cantharides [177], mercaptoethanol [178], benzodiazepine [179]. Likewise, GC–MS has been instrumental in clarifying cases of chemical and pesticide poisoning, as for instance with cresol [180], malathion [181], diuron [182], the latter having also been identified in human postmortem plasma and urine by HPLC–MS with a moving-belt interface [183]. Also, though its toxicological significance in the sample is not clear at present, GC–MS was used to detect the presence of pentachlorophenol in oil samples associated with the Spanish toxic oil syndrome [184].

FABMS and GC–MS were used to identify PAF and PGD_2, respectively, in blister fluid from a case of bullous mastocytosis in an infant with congenital onset of the disease [185]. In another study, stable isotope dilution GC–MS was used to determine patterns of episodic diurnal secretion of cortisol in three normal subjects and four paediatric patients with orthostatic dysregulation [186]. As claimed by these authors, the daily cortisol profile could serve as a biochemical index for differentiating diagnostic types of orthostatic dysregulation.

A further interesting example in this area is the quantitative electron capture NI–MS assay for dexamethasone in human plasma of psychiatric patients undergoing the dexamethasone suppression test [187]. As indicated, the unique and novel feature of this assay is the chemical oxidation of the sample, which transforms dexamethasone into a highly electrophilic species. This affords a significant improvement in sensitivity so that sample size can be reduced to 50 times less than that required for GC–MS. An interesting application of LSIMS has been described by Guo et al. [188] who isolated and identified β-hydroxypropionic acid-uroporphyrin I in the urine of a patient with congenital erythropoyetic porphyria. Finally, laser microprobe mass spectrometry has been applied to a study of the chemical composition of

spheroliths in the Bowman's membrane of two patients suffering from primary atypical bandkeratopaphy [189]. The inclusions in the corneal biopsy appeared to consist mainly of calcium phosphate.

IN VITRO STUDIES

Work in vitro is very widespread across different fields of biochemistry and biomedicine although, as indicated in Table 6, on the average it represents a relatively low percentage compared with the number of reports on human or animal studies. However, as also indicated in this table, in vitro work with human material is even more restricted. Nevertheless, a few interesting applications of mass spectrometry in in vitro studies on human or related cell culture systems have appeared in the more recent literature.

For example, cholesterol extracted from human fibroblasts and hepatoma cells grown in media containing 25% D_2O and the bile acids from the media have been analysed by GC–MS [190]. Results indicated that deuterium incorporation occurs randomly. The analysis of chenodeoxycholic acid shows that it derives from both pre-formed and newly synthesized cholesterol and that a ring transformation from cholesterol utilizes deuterium derived from water. Cultured fibroblasts have also been used in the study of a patient presenting a familial giant cell hepatitis and who was excreting 3β-hydroxy-δ-5-bile acid. The results, as indicated in the previous section on diagnosis, disease, biochemical and biological markers, suggested that the patient's liver disease was due to a primary defect in a dehydrogenase/isomerase enzyme involved in bile acid biosynthesis [191]. GC–MS was likewise used in the study of androgen and odorous 16-androstene metabolism by human axillary bacteria from 34 men which were selectively cultured for aerobic coryneform bacteria [192].

Another area which has attracted some attention are the studies on leukotriene production by human cells, such as for example the neutrophile. In this regard a GC–MS study explored the origin of the arachidonate used by the human neutrophile for leukotriene biosynthesis [193]. Arachidonate was assayed by SIM as the corresponding *t*-butyldimethylsilyl ester derivative whereas in another study, also in human neutrophiles, LTB4 was analysed by NICI–SIM of the pentafluorobenzyl ester trimethylsilyl ether derivative [194]. GC–MS–SIM with a mass-selective detector has been used also for the determination of LTB_4 as the methyl ester, bis-*t*-butyldimethylsilyl ether derivative in human plasma. The method which gave a limit of detection of 425 pg allowed the study of a new anti-inflammatory drug on ionophore stimulated LTB_4 biosynthesis by human whole blood in vitro [195]. Likewise, human neutrophile cultures have been used for the quantification of PAF by FABMS–MS with deuterium-labelled internal standard [196] and more recently, on-line thermospray HPLC–MS has been demonstrated to facilitate

the identification of individual glycerophospholipid molecular species in preparations of human erythrocytes. Identifications are possible even when more than one molecular species is contained in an HPLC peak [197].

HIGH MASS ANALYSES

In view of the truly dramatic developments we have witnessed in the last 3 years regarding the ability of new ionization techniques to provide molecular mass assignments well into the 200 000 u range or over, a specific section devoted to high mass analysis (above 1000 u) seems warranted. These developments, which mainly encompass LD and ES ionization techniques, have already shown their potential in contributing to the structural characterization of biopolymers and to macromolecular MS in general. A good account of their advantages and limitations can be found in ref. 3. However, with a few exceptions, as discussed below, the relatively few applications of high mass MS to clinical and biomedical studies are limited to the more established FAB, LSIMS and occasionally PDMS techniques, allowing studies of biopolymers with a molecular weight of up to around 15 000 u or less. Nevertheless, recent reported applications mostly cover the areas of metabolism of endogenous compounds, biomarkers, clinical and in vitro studies.

A very active field where the more classical techniques combine with the latest ionization techniques to produce results not considered within the realm of possibilities just a decade ago lies in the characterization of abnormal human haemoglobins. The more recent work on haemoglobins deals with characterization of normal vs. a modified haemoglobin C, an 0-Arab haemoglobin Indianapolis by FAB mapping techniques [198,199]. The same authors have gone on with these techniques to identify two more haemoglobin variants, HbM Hyde Park and Hb San Jose; the first is characterized by substitution of the glutamic acid residue by a glycine at position 7 of the β chain and in the last the histidine residue at position 92 also in the β chain is substituted by a tyrosine. Although MS analysis is usually carried out on the peptide mixture generated by tryptic digestion of the abnormal globin, in this case a V-8 protease was used because the tryptic map alone was unable to locate the modification unequivocally [200,201]. A V-8 protease was also used by Prome et al. [202] for the identification of the so-called R and Grenoble variants by FAB mapping. The same group also applied these MS techniques to characterize the structure of a mutant 89Arg· · ·Cys biphosphoglycerate mutase isolated from a patient in a previously reported case. Their results indicated that Arg89 is at or near the active site of the enzyme and probably involved in the binding of the monophosphoglycerate [203].

Clinical diagnosis of variant haemoglobins has been recently achieved by ESMS [204,205]. As indicated in these reports, ESMS allowed analysis of

mixtures of intact globins, giving molecular weights and information on the relative amounts of globins present. The minimum molecular weight difference to measure accurately different species present in a mixture of 15–16 kDa proteins is at present 14 u. Experimental values for the molecular masses of globins can be approached to within 0.5 Da of values calculated from the amino acid composition. Thus ESMS can be highly recommended in the initial steps of abnormal haemoglobin detection and identification and should become in the opinion of these authors a valuable preliminary diagnostic procedure for the detection and partial molecular identification of variant haemoglobins. ESMS has also been very recently used for the characterization of opioid peptides in human pituitary extracts [206]. The motivation for exploring the use of ES in this case lies in the difficulty in ionizing many of the endogenous neuropeptides and in the interferences at the picomole level because of the chemical noise derived from the FAB matrix. Both methionine enkephalin (ME) and β-endorphin (BE) were quantified by ES, with detection limits of the order of 1 pmol for ME and 5 fmol for BE, which was 17 times more sensitive than FABMS. A further clear advantage of ES over FAB is that because the molecular weight of BE (3463 u) is beyond the mass range of many spectrometers it becomes more accessible to monitor multiply charged ions as produced by ES. However, ES spectra are for the most part devoid of sequential information unless MS–MS of sequence-specific ions can be performed, as proposed by Huang and Henion [207]. These authors contend that on-line electrospray HPLC–MS detection is a convenient and preferred approach to conventional HPLC and FAB tryptic mapping.

In this fashion it becomes possible to determine the molecular mass to within 1 u for each tryptic fragment separated on line by microbore HPLC. With this data on hand, sequence or structural information based on the formation of CAD product ions in a tandem assay becomes a matter of proper experimental parameter set-up.

Opioid and tachykinin peptides were determined in human CSF by a combination of techniques, including FABMS B/E linked-field scan of $(M + H)^+$ ions which provided the fragment ions necessary to corroborate amino acid sequences [208]. High sensitivity tandem mass spectrometry has been applied to the characterization of structural xenobiotic modifications in human haemoglobin [209]. In this case haemoglobin was modified in vitro with styrene 7,8-oxide, a xenobiotic known to be both mutagenic and carcinogenic in rodents, and digested with trypsin. High performance MS–MS of modified tryptic peptides allowed unambiguous assignment of the specific residues modified. A later report from the same group describes the detection of styrene-oxide DNA adducts in workers exposed to styrene [210].

Alveolar neumocytes secrete a complex phospholipid protein mixture which acts as a primary surfactant. One of these proteins with a molecular

weight of 4000 u reduces alveolar surface tension, and owing to its extreme hydrophobicity protein-analytical methods have proved difficult for its study. However, combined selective/Edman degradation reactions, ^{252}Cf PDMS and FABMS have been successfully used to elucidate the complete primary structure [211]. PDMS has also been shown to be of use in the direct identification of epitope peptides. In this regard limited enzymatic proteolysis combined with PDMS has been applied to the molecular epitope analysis of the complement component C3a, a potent mediator of inflammation [212].

The determination of complex carbohydrates which are responsible for the modulation of biological responses in living systems mediated by processes of biopolymer glycosylation (lipids and proteins) has benefited greatly from the recent introduction of new soft ionization procedures, such as LDMS [213]. However, most studies on human material to date have been carried out by FABMS, as described in some detail by Burlingame and co-workers [214] in the characterization of a series of high mannose oligosaccharides isolated from human IgM purified from a patient with Waldenstrom's macroglobulinemia. For instance, the structures of human skim milk or amniotic mucins could be established by FAB and EIMS combined with methylation analysis, NMR and *endo-β*-galactosidase digestion [215,217]. Interest in the structural and functional aspects of carbohydrates carried by mucins has been sparked by their possible use in the diagnosis of a variety of human tumours. Interesting work on human glycoproteins has been described by Lawson and co-workers [218–220] who have used a combination of techniques, including GC–MS and LSIMS to identify several disaccharides to hexasaccharides plus a novel oligosaccharide backbone structure with a galactose residue monosubstituted at C6. Another group has also resorted to LSIMS, MS–MS and Edman sequence analysis to characterize O-glycosylation sites in recombinant human platelet derived growth factor β chain, confirming the complete sequence [221]. A comprehensive FABMS approach to the isolation and structural analysis of oligosaccharides derived from glycoproteins has been recently described [222]. Along these lines, the recent work of Reinhold and co-workers [223–227] and Fournet and co-workers [228] is worth mentioning as these authors have shown the advantages of supercritical fluid chromatography–mass spectrometry in the study of carbohydrate structures.

High molecular weight glycolipids are also amenable to soft ionization techniques and very recently Curtis et al. [229] have described the use of four-sector EI-tandem MS for the analysis of two blood group Le b and B hexaglycosylceramide isomers from human small intestine and pancreas as well as for the identification of a hexaglycosyl-ceramide in a glycolipid mixture isolated from a liver from a blood group A1Le(a-b+) human individual transplanted into a blood group O recipient. Although this application illustrates the utility of direct four-sector MS without on-line chromatography it

seems that sensitivity is somewhat disappointing even though, as indicated by the authors, this could be solved by the use of electro-optical multichannel array detection systems.

Finally, a novel sulphated glycosphingolipid has been recently isolated from the human kidney and characterized in part by SIMS [230], whereas FABMS has been instrumental in providing the structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase [231].

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