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Applications of gas chromatography–mass spectrometry in clinical chemistry

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Abstract

Two applications of gas chromatography–mass spectrometry in clinical chemistry, are described, namely the identification and determination of compounds present in biological fluids. For the first application, the biological substances are derivatized to stable forms for gas chromatography and to give appropriate spectra in the mass spectrometer operated in the scanning mode. This method is used for the diagnosis of organic acidurias, different enzyme deficiencies causing congenital adrenal hyperplasia and other disturbances of steroid metabolism, for differentiation between adrenal carcinoma or adenoma, etc. The second application is the use of the mass spectrometer as a very sensitive and selective gas chromatographic detector. This, in combination with stable isotope dilution methods, is the most accurate analytical method in clinical chemistry. The substances are derivatized to forms suitable for gas chromatography and to give a few specific and intense fragment ions in the mass spectrometer operated in selected-ion monitoring mode. Substances that can be detected in this way include steroids, vitamins, prostaglandins, carbohydrates, drugs and pesticides.

1. Introduction

Gas chromatography–mass spectrometry (GC–MS) was, in earlier times, a method that needed very skilled laboratory staff and was therefore used only in some specialized centres. More recently GC–MS has become easier to handle and the instruments smaller and it is now widely used in clinical chemistry. The development of very sensitive desktop instruments enables the user to measure, in the electron impact (EI) or chemical ionization (CI) mode, positive and negative ions in the scan mode or with selected-ion monitoring (SIM). This has greatly widened the applications of GC–MS. MS has some great advantages over other GC detectors. Different metabolites in a chromatogram are identified by other GC detectors only from the retention time previously detected with standards. Using GC–MS, the substances are identified on the basis of their spectra and a computerized library search. Moreover, substances can be localized by specific ion chromatograms [1]. In this way, it became possible to install computer programs carrying out the diagnosis with respect to retention times and spectra of the key metabolites of a certain syndrome. This is especially useful in the diagnosis of inborn errors of amino acid metabolism, where the interpretation of a urinary chromatogram would be laborious. Quantification can be achieved using GC–MS and stable isotope dilution (SID) techniques [2]. Substances labelled with stable isotopes have nearly the same chemical and physical properties as their natural analogues but can be detected by MS in the presence of the natural analogues

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from the difference in molecular mass. The labelled analogue is added in a constant amount to the sample immediately after collection. After the entire preparation steps, the substances are converted into derivatives giving a few specific and prominent fragment ions in the MS ion source. The mass spectrometer is operated in the SIM mode in order to obtain the highest possible sensitivity. The labelled compound is measured simultaneously with the natural analogue. All loss is compensated for by the calculation based on the comparison of the peak area of a specific ion of the labelled substance with that of the corresponding ion of the natural metabolite. SID–GC–MS is the most sensitive and accurate analytical method in clinical chemistry [3] and can be applied to all biological substances that are thermally stable enough for GC.

2. Experimental

2.1. Materials

N,N-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), heptafluorobutyric anhydride (HFBA), acetonitrile and pyridine were obtained from Pierce (Rockford, IL, USA), pentafluorobenzyl bromide (PFB-Br) from ICT (Vienna, Austria), [^{18}O]water and platinum dioxide from Ventron (Karlsruhe, Germany), Silicar CC4 was from Mallinckrodt (St. Louis, MO, USA), *tert.*-butyldimethylsilyl (tBDMS) chloride from Fluka (Buchs, Switzerland) and β-glucuronidase–aryl sulphatase from Serva (Heidelberg, Germany). All other reagents and solvents of analytical-reagent grade were purchased from Merck (Darmstadt, Germany).

2.2. Gas chromatography

Analysis was performed on a Fisons GC 8000 gas chromatograph coupled to a Trio 1000 quadrupole mass spectrometer (Fisons Instruments, Manchester, UK). The gas chromatograph was equipped with a DB-5 capillary column (15 m × 0.25 mm I.D., 0.25-μm film thickness) (Fisons Instruments). Helium was used as the carrier gas. The splitless Grob injector was kept at 290°C. The temperature programme of the column was adapted to the different classes of substances so that minimum retention times and optimum separations were achieved.

2.3. Mass spectrometry

EI and CI mass spectra were recorded with one scan per second; the mass range was adapted to the particular needs of the substance classes. In the SIM mode the sampling time was set at 100 or 200 ms per mass. Positive-and negative-ion CI mass spectra were measured with methane as reagent or moderating gas and an electron energy of 70 eV. The GC–MS transfer line was kept at 310°C and the ion source temperature was 220°C.

2.4. Derivatization

For silylation, 50 μl of BSTFA–pyridine (1:2, v/v) were added to the dried sample, after heating at 75°C for 30 min the reagent was removed by evaporation under a stream of nitrogen and the residue was dissolved in 50 μl of hexane [4].

Pentafluorobenzyl (PFB) esters were prepared by reaction with 50 μl of PFB-Br solution in acetonitrile (7%, w/w) and 10 μl of diisopropylethylamine for 15 min at room temperature [5].

To prepare heptafluorobutyrates, 100 μl of HFBA–benzene–triethylamine (10:200:1, v/v/v) were added to the dried sample and reacted at 75°C for 30 min., 200 μl of benzene were added and the mixture was washed with 1% ammonia solution and then with water. The organic phase was dried under nitrogen and dissolved in hexane or dichloromethane [6].

2.5. Urine analysis for steroids

The steroids excreted in the urine as glucuronides and sulphates were hydrolysed enzymatically with β-glucuronidase–aryl sulphatase at pH 5 and 37°C for 30 h. The pH was adjusted to 6 and the sample brought on to an Extrelut 20 column. The steroids were eluted with 40 ml of

dichloromethane. The eluate was evaporated to dryness and the residue dissolved in 5 ml of benzene. A preseparation was performed on 4 g of alumina washing with 30 ml of benzene and eluting with 20 ml of benzene–ethanol (8:2). The extract was dried and than silylated [7].

2.6. Urine analysis for organic acids

The analysis of the pattern of organic acids in urine is very useful for the diagnosis of inborn errors of amino acid metabolism [9,10]. However, the preparation of the urine is a major problem. Many substances with very different polarities, such as sugars, amino acids, phosphoric acid, urea, steroids and bile acids, are present in the urine and the polarities of the different organic acids differ widely. Various methods for the separation of the organic acids from other urinary compounds have been described [11], but in our experience part of the organic acids is lost in these preseparation steps. We therefore used the following procedure: deuterated methylmalonic acid, deuterated N-acetylaspartic acid and tricarballylic acid were added as internal standards to a part of the fresh urine corresponding to 1 μmol of creatinine. After freeze-drying, the residue was silylated and a part was injected on to the GC column. The temperature programme was 1 min at 40°C, then increased at 5°C/min to 180°C and at 30°C/min to 310°C.

3. Results and discussion

The use of GC–MS for the elucidation of the pattern of metabolites is demonstrated on the analysis of urine from a patient with congenital adrenal hyperplasia (CAH) and a patient with an inborn error of amino acid metabolism, namely propionic acidaemia. GC–MS for the determination of substances by use of SID–GC–MS is shown for the measurement of the urinary excretion of N-acetylaspartic acid from a patient with Canavan's disease.

3.1. Identification of urinary steroids

The analysis of the urinary steroid pattern was used for the diagnosis of different enzyme deficiencies in adrenal cortisol production.

Patient 1. Fig. 1 shows the results obtained form a 3-month-old girl suspect to have CAH. In the total ion chromatogram four prominent peaks were obtained. The two peaks at m/z 360 and 270 are significant fragments of 17-oxo-

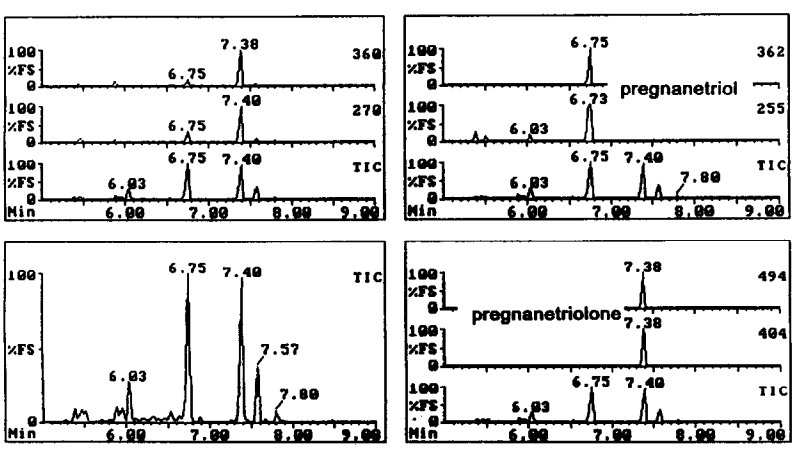

Fig. 1. Urine extract form a girl with congenital adrenal hyperplasia. The total ion chromatogram (TIC) and specific ion chromatograms together with the TIC are shown together but with four different windows. The ions at m/z 360 and 270 are usually indicative of androsterone and etiocholanolone; in this extract both steroids are absent and the two peaks result from unspecific fragments of pregnanetriol and pregnanetriolone, as the retention times correspond to those of the ions at m/z 362 and 255 characteristic of pregnanetriol and m/z 494 and 404 specific for pregnanetriolone.

steroids, but in this urine extract the two intense peaks in these ion chromatograms were identified as ions of key metabolites of a 21-hydroxylase deficiency, namely pregnane-3,17,20-triol and pregnane-3,17,20-triol-11-one. This is demonstrated through the ion chromatograms with m/z 362 and 255 corresponding to pregnanetriol and m/z 494 and 404 resulting from pregnanetriolone and identification from the spectra and a library search. The peak with retention time (t_R) 6.03 min was identified as pregnane-3,20-diol and that with $t_R = 7.57$ min as pregn-5-ene-3,17,20,21-tetraol-11-one. Substantial amounts of 17-oxosteroids were not detectable in this urine extract. The absence of this group of urinary steroids may be due to a multiple adrenal enzyme deficiency. The ability to reveal the presence or absence of metabolites from the mass chromatograms makes the interpretation of a chromatogram easy to understand even by untrained workers. Additionally, based on the spectra, unusual steroids can be identified.

Patient 2. An extremely tall girl was suspected of cortisol overproduction. The urinary steroid chromatogram showed a very high peak which was identified as dehydroepiandrosterone (DHEA). This metabolite is indicative of an adrenal carcinoma [8] and surgical intervention showed the tumour, which could be removed. In this instance the use of GC–MS was crucial as surgical intervention was recommented based only on the presence of a high excretion of DHEA, which was identified from the retention time and spectrum.

3.2. Evaluation of the organic acid pattern in urine

The evaluation of the often very complex chromatograms is done by a computer program. Three organic acids, key metabolites of the disease, are selected for each of the most common organo acidurias. These metabolites are detected in a time window through two specific fragments. Some organo acidurias are listed in Table 1 together with three key metabolites, their two specific fragment ions and the relative retention times (t_R). Fig. 2 shows the result of the use of such a computer program for a patient with a severe form of propionic acidaemia. In this instance most of the compounds present in the urine remain invisible in the total ion chromatogram (TIC), suppressed by the extremely high amounts of lactic acid, 3-hydroxypropionic acid and 3-hydroxybutyric acid. The printout shows in four windows the TIC and the mass chromatograms of the three main organic acids, lactic acid [time window (tw) 7–9 min, m/z 190 and 219], 3-hydroxypropionic acid (tw 10–12 min, m/z 177 and 219) and 3-hydroxybutyric acid (tw 10–12 min, m/z 191 and 117). These three substances are rapidly identified from the spectra and a library search (Fig. 3) and the disease can be diagnosed. A good correlation is obtained for the library search irrespective from the fact that the amount of 3-hydroxypropionic acid was too high for the dynamic range of the multiplier.

Quantification of the more important metabolites can be achieved with the aid of a computer by correlation of the peak areas of specific masses of the substance of interest with those of the internal standards, which are m/z 221 for [$^2H_3$]methylmalonic acid, m/z 307 for [$^2H_3$]-N-acetylaspartic acid and m/z 377 for tricarballylic acid. The computer program used allows the rapid identification of the key metabolites also in complex chromatograms and helps to provide confidence in the presence or absence of expected substances and hence to avoid a wrong diagnosis.

3.3. Determination of metabolites by SID–GC–MS

Substances labelled with stable isotopes such as deuterium or oxygen-18 (^{18}O) show nearly the same chemical and physical behaviour as their natural analogues but can be detected simultaneously with the natural products by MS. Substances labelled with stable isotopes are therefore ideal internal standards for GC–MS procedures. They are added in a constant amount to the biological sample immediately after collection and follow the whole preseparation and derivatization process. Quantification is per-

Table 1
Organic acidurias, key metabolites, specific fragments and relative retention times

Disease	Specific metabolite	m/z	m/z	t_R
Propionic acidaemia	3-Hydroxy-propionic acid	219	177	115
	Lactic acid	219	190	106
	3-Hydroxy-butyric acid	191	117	116
Methylmalonic acidaemia	Methylmalonic acid	247	218	122
	3-Hydroxy-propionic acid	219	177	115
	3-Hydroxy-isovaleric	205	131	121
Malonic aciduria	Malonic acid	233	133	121
	Methylmalonic acid	247	218	122
	Succinic acid	247	172	131
Isovaleric acidaemia	3-Hydroxy-isovaleric acid	205	131	121
	3-Hydroxy-isovalerylglycine	261	176	152
	4-Hydroxyl-isovaleric acid	204	247	126
Methylglutaconic acidaemia	3-Methylglutaconic acid E	273	198	148
	3-Methylglutaric acid	204	69	143
	3-Hydroxy-isovaleric acid	205	131	121
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency	3-Hydroxy-3-methylglutaric acid	363	273	161
	3'-Methylglutaric acid	204	69	143
	3-Hydroxy-isovaleric acid	205	131	121
Glutaric acid type 1	Glutaric acid	261	158	140
	3-Hydroxy-glutaric acid	259	217	158
	Glutaconic acid	259	217	145
Glutaric acid type 2	Glutaric acid	261	158	140
	Ethylmalonic acid	261	217	129
	Adipic acid	275	111	151
Fumarase deficiency	Fumaric acid	245	147	135
	Succinic acid	247	172	132
	Lactic acid	219	190	106
Canavan's disease	N-Acetylaspartic acid	304	158	167
Quantification with	$[^2H_3]$-N-Acetylaspartic acid	307	161	167

formed by comparing the peak area of a specific fragment of the labelled compound with that of the corresponding fragment of the natural analogue. The substances can be measured in the EI, CI or negative-ion chemical ionization (NICI) mode, but multiple ion detection (MID) has to be used for exact quantification. All loss occurring during the preparation of the sample is compensated for in this way. This method is therefore the most accurate method in clinical chemistry. Measuring in the NICI mode is often also the most sensitive quantification procedure. Some substances measured in our laboratory in the NICI mode are listed in Table 2 together with detection limits and relative standard deviations (R.S.D.s).

The results of the analysis of the urinary excretion of N-acetylaspartic acid by a patient with Canavan's disease are given as an example of quantification in the EI mode. Canavan's disease is a form of leukodystrophy inherited as an autosomal recessive disorder combined with spongy degeneration of the brain. The aspartoacylase deficiency [12,13] causes a highly increased level of urinary N-acetyl-L-aspartic acid, which is used for diagnosis. A 200 n*M* concentration of $[^2H_3]$-N-acetylaspartic acid [14] was added to an amount of urine corresponding to 1

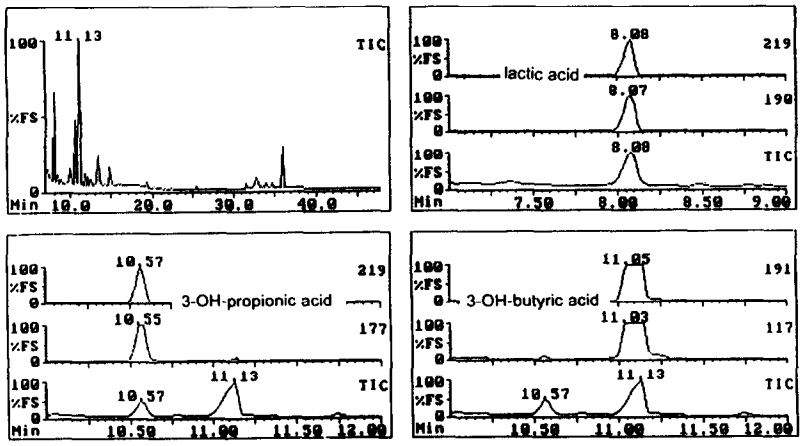

Fig. 2. Analysis of freeze-dried urine from a patient with propionic acidaemia after silylation. One window shows the TIC of the whole run and the others show the ion chromatograms of two specific ions of three key metabolites of the disease together with the TIC in the corresponding time window (tw), namely lactic acid (tw = 7–9 min, m/z 219 and 190), 3-hydroxypropionic acid (tw = 10–12 min, m/z 219 and 177) and 3-hydroxybutyric acid (tw = 10–12, m/z 191 and 117).

μM creatinine, than it was freeze-dried, silylated and injected splitless on to the GC column. The temperature program was 1 min at 80°C, then increased at 30°C/min at 330°C, where it was maintained for 10 min. The chromatogram in Fig. 4 was obtained in the EI mode scanning form m/z 40 to 500 with one scan per second. The spectrum on the left was obtained in the same way from N-acetylaspartic acid, whereas that on the right was obtained from the peak resulting from natural and [$^2H_3$]-N-acetylaspartic acid present in the sample. The fragments of the natural substance can be readily observed beside the fragments of the triply labeled compound. The influence of the kinetic isotope effect is demonstrated by the differences in the retention times of the labelled and unlabelled analogues. Deuteration shortens the retention times slightly, thus the retention time of the labelled N-acetylaspartic acid TMS derivative was 9.18 min (m/z 307 and 161) compared with 9.20 min for the natural analogue (m/z 304 and 158). Quanti-

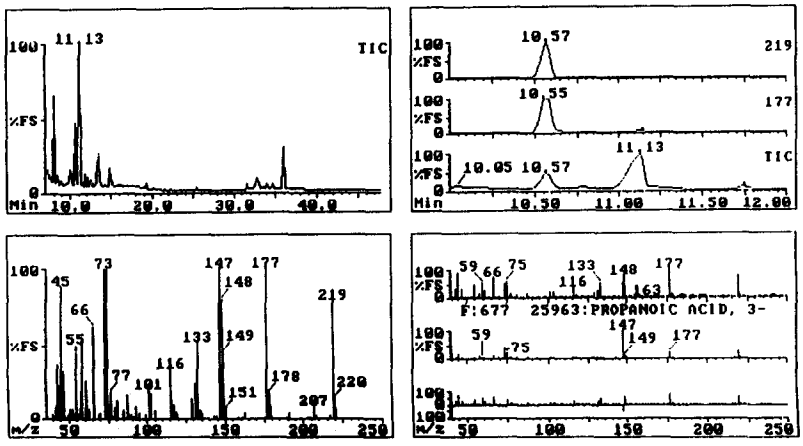

Fig. 3. Analysis of freeze-dried urine from a patient with propionic acidaemia after silylation. The four windows show the TIC, the ion chromatograms of m/z 219 and 177 in the time window 10–12 min characteristic of 3-hydroxypropionic acid, the spectrum of the peak with a retention time of 10.55 min and the results of the library search.

Table 2
Detection limits and variability for SID–GC–NICI-MS determination different compounds

Substance	Ref.	Detection limit (pg/per sample)	R.S.D. (%)[a]	
			Intra-assay	Inter-assay
Prostaglandins (PGs)	15			
$PGF_{2\alpha}$		5	1.1	2.4
PGE_2		20	2.2	2.3
PGE_1		40	1.3	2.3
6-Keto-$PGF_{1\alpha}$		30	1.4	2.0
Thromboxanes (TX_s)	15′			
TXB_2		20	2.0	2.2
2,3-dinor TXB_2		10	1.1	2.9
Hydroxy fatty acids	16			
5-HETE[b]		40	2.8	3.9
12-HETE		40	2.8	4.0
15-HETE		15	2.7	3.9
12-HHT[b]		10	2.9	3.9
Drugs				
Diclofenac	17	10	0.34	4.04
Terbutaline	18	100	0.95	1.35
Orciprenaline	18	100	2.14	2.04
Captopril	19	500	1.15	2.13
Ketotifen	20		2.3	4.5

[a] $n = 10$.
[b] HETE = Hydroxy-eicosa-tetraenoic acid; HHT = hydroxy-heptadeca-trienoic acid.

fication was carried out by another GC run where the mass spectrometer was again operated in the EI mode but with MID. For the $[^2H_3]$-N-acetylasparate and ion at m/z 307 was recorded for quantification and that at m/z 161 for identification. For the natural analogue the ion for

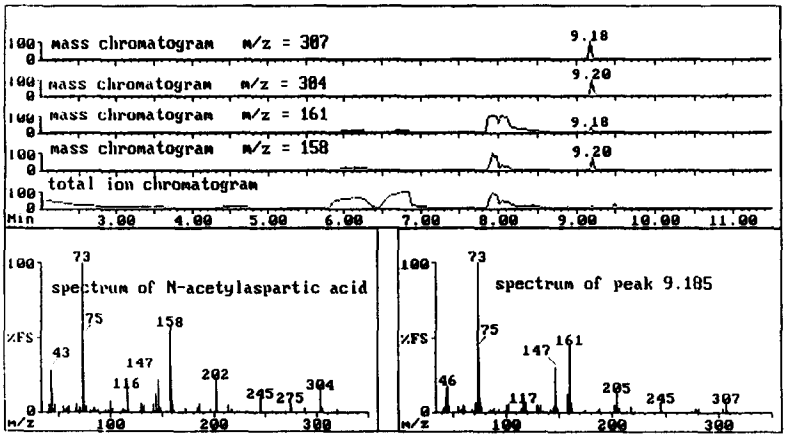

Fig. 4. Analysis of freeze-dried urine form a patient with Canavan's disease after silylation. The three windows show the TIC together with the ion chromatograms of m/z 304 and 158 indicative of natural N-acetylaspartic acid and m/z 307 and 161 corresponding to the trideuterated analogue. One window shows the spectrum of natural N-acetylaspartic acid and one that of the peak with a retention time of 9.185 min.

quantification was that at m/z 304 and the ion at m/z 158 was used for confirmation. An excretion of 75 μM of N-acetylaspartic acid per mM of creatinine was found. The level in healthy persons is *ca.* 3 μM per mM creatinine, so the diagnosis of Canavan's disease was confirmed by the fifteenfold elevated excretion of N-acetylaspartic acid. This is a good example demonstrating the use of GC–MS in both modes for the identification of a key metabolite and quantification by SID measurement.

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