Journal of Chromatography, 489 (1989) 353–358
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4639

Note

Screening of amniotic fluid metabolites by gas chromatography–mass spectrometry

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(First received September 29th, 1988; revised manuscript received December 8th, 1988)

The principal methodological approach to the prenatal diagnosis of hereditary metabolic diseases consists in determining the activity of those enzymes which are presumed to be deficient in cultured fibroblasts from the amniotic fluid (AF). This approach is selective with respect to each known metabolic defect and it requires specific, often costly, reagents and laboratory equipment and experience in detecting the disturbed activity of a particular enzyme. In addition, one cannot determine the activity of all enzymes in fibroblasts. To obtain a sufficient amount of fibroblasts for enzymatic analysis, normally cultivation for at least three weeks is required, provided that the growth is good. Of course, this selective approach cannot be used at all for prenatal diagnosis in those numerous cases where the deficient enzyme is unknown.

With regard to hereditary metabolic diseases in which the products of disturbed metabolism are released by cells and subsequently enter the urine, the above limitations of prenatal diagnosis can be avoided through the determination of AF components, because from the fourteenth week of pregnancy the AF chiefly contains the components of the foetus's urine. Although this provides potential possibilities for the prenatal diagnosis of a large number of metabolic disturbances, mostly of the organic aciduria type, only a few cases have been described [1,2]. To establish which metabolic defects can be diagnosed through the analysis of AF, one has to know which metabolites find their way into it. The composition of the AF metabolites must be examined by non-selective methods, allowing the screening of metabolites or metabolic profiling for a wide range of compounds.

0378-4347/89/$03.50

The most effective way of screening metabolites in human body fluids is gas chromatography coupled with mass spectrometry (GC–MS) [3]. The organic acid profiles of AF have been obtained by GC–MS in several studies [1,4,5]. About 40 metabolites were recorded in the form of trimethylsilyl (TMS) derivatives through the use of packed chromatographic columns. In this work, for the screening of both acidic and neutral non-volatile AF metabolites, we used capillary GC–MS, allowing 390 metabolites to be recorded in ten AF samples.

EXPERIMENTAL

Sample preparation

AF was obtained by transabdominal amniocentesis from ten women on whom amniocentesis had been carried out with a view to diagnosing chromosomal anomalies and the adrenogenital syndrome of the foetus; no pathology was detected in any of the cases and all the newborns were healthy. Thus all the AF samples examined were associated with uncomplicated pregnancies. The term of pregnancy at the time the AF samples were obtained was 20 weeks on average (range 18–23). Immediately after the amniocentesis 10 ml of AF were isolated from the cells by centrifugation and the samples were prepared and analysed within 1 h. The samples were prepared as follows: sodium chloride was added up to saturation to 10 ml of AF, the pH was adjusted to 1 with 5 *M* hydrochloric acid, then 20 ml of distilled ethyl acetate were added, followed by vigorous shaking for 5 min; after centrifugation, for a better separation of the aqueous and ethyl acetate fractions, the latter was removed and the aqueous fraction was re-extracted with ethyl acetate; the ethyl acetate extract of AF was evaporated to about 2 ml on a rotary evaporator at 40°C, then the remainder was transferred into a micro-reaction flask and dried completely at 60°C in a current of dry purified air, then re-dried after adding 1 ml of spectroscopically pure acetone. The flask was sealed and 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rockford, IL, U.S.A.) were introduced through the septum, then the sample was kept at 60°C for 40–60 min prior to analysis.

Gas chromatography–mass spectrometry

The samples were analysed by means of a Hewlett-Packard 5995B GC–MS system with a quadrupole mass filter and a gas chromatograph with a special 'ion-trap' mass spectrometric detector (Finnigan-MAT 700). The conditions of chromatography were the same for both instruments. We used fused-silica capillary columns with cross-linked 5% phenylmethylsilicone (Hewlett-Packard, Ultra performance series): column 25 m$\times$0.31 mm I.D., film thickness 0.52 μm, performance 3000 plates/m. The temperature programme was as follows: 100°C for 5 min, then increased at 5°C/min to 290°C and held at 290°C for 15 min. The carrier gas was helium at a flow-rate of 1.5 ml/min and the injector temperature was 250°C. A sample inlet system with a 1:10 split (for both instruments) was used; the sample volume was 1 μl. The ion source temperatures were 220°C (Hewlett-Packard) and 270°C (Finnigan-MAT) and the scanning rates were

690 and 650 a.m.u./s, respectively. The sensitivity of both instruments was maintained at the constant optimum level by means of the Autotune system.

The result of the metabolite screening for each sample is a chromatogram of the TMS derivatives of AF metabolites recorded by the total ion current, specifying the retention time, the size of the chromatographic peak in total ion current units and the mass spectrum of the compound corresponding to the chromatographic peak. The registered compounds were identified through a computer comparison of the resulting mass spectra with the library of mass spectra of the U.S. National Bureau of Standards [6]. Standard compounds were also used in some questionable cases.

RESULTS AND DISCUSSION

Having analysed AF samples from ten individuals, we recorded the characteristic retention times and mass spectra of 390 compounds. The number of metabolites detected in individual samples was 120 on average. Fig. 1 presents the ion chromatogram of metabolites (in the form of TMS derivatives) for one of the AF samples obtained in the eighteenth week of uncomplicated pregnancy. This chromatogram was recorded by means of the Finnigan-MAT 700 ion-trap detector. There was a good correlation between the chromatograms of the same sample obtained with the Hewlett-Packard 5995B GC–MS system and the Finnigan-MAT 700 ion-trap detector. The mass spectra showed certain differences between the two instruments. The ion-trap detector recorded more high-intensity peaks in the m/z 40–70 range. It seems reasonable, therefore, to use this instrument for recording mass spectra starting at m/z 70, especially if one uses the Eight Peak Index of Mass Spectra [7] for their identification.

The fact that each individual sample exhibited about a third of all the metabolites recorded in ten samples does not mean that there are large qualitative differences between the various AF samples. It is more likely that all the 390 metabolites are present in each sample, but two thirds are at concentrations below the detection level. According to the chromatograms, the most stable metabolites in the AF samples are also present in the largest average amounts. Table I lists these metabolites together with their retention times in methylene units and total ion current values.

Our data on the most frequently occurring and most abundant AF metabolites are in agreement with the data reported in the literature [1,4,5]. The only exception is pyruvate, which may not have been detected because we did not treat the samples with hydroxylamine so as to produce keto acid oximes as we were looking for methoxyacetylcarbamide, which we had previously identified in the urine of phenylketonuric children and normal adults [8] and which is destroyed by hydroxylamine. In fact, we did not find this metabolite in AF.

Table I shows that lactic acid is the major metabolite in AF. Together with this, the presence of relatively large amounts of succinic, 3-hydroxyisobutyric, 3-hydroxybutyric fatty acids and cholesterol can be interpreted as a manifestation of the lactacidosis of the foetus, associated with the peculiar metabolism of fatty

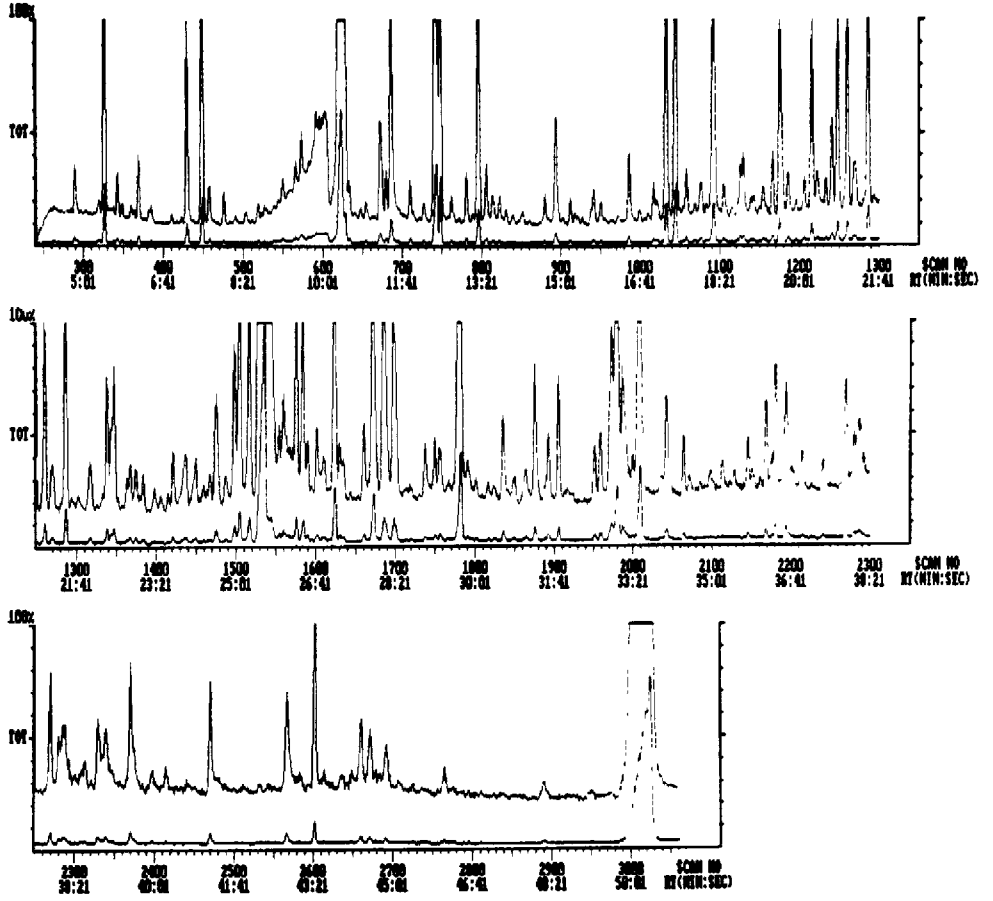

Fig. 1. Gas chromatogram of AF metabolites in the form of TMS derivatives recorded by the total ion current. The metabolites were extracted with ethyl acetate from 10 ml of AF obtained at eighteenth week of pregnancy. The chromatogram was recorded using a Finnigan-MAT 700 gas chromatograph with an ion-trap mass spectrometric detector. Chromatographic conditions: fused-silica capillary column (25 m × 0.31 mm I.D.) with cross-linked 5% phenylmethylsilicone, film thickness 0.52 μm; column temperature, 100 °C for 5 min, increased at 5 °C/min to 290 °C, then held at 290 °C for 15 min.

acids and cholesterol. Note that cultured human skin fibroblasts also excrete large amounts of lactic acid and cholesterol [9].

The total ion current abundance is a quantitative characteristic of metabolites. It characterizes the size of the chromatographic peak, but fails to take into account the metabolite's extractability by ethyl acetate and certain differences in the detection efficiency for individual compounds. Neither of these factors, however, affects the quantitative proportions of peaks in a metabolic profile. If one knows the peak proportions of the metabolite profile for AF or some other physiological fluid in the normal organism, one can detect metabolic disorders by the relative increase in size of a particular peak. If a metabolite that exceeds the maximum normal level or is absent from the normal fluid has not been identified,

TABLE I

MOST CHARACTERISTIC METABOLITES OF AMNIOTIC FLUID IN THE FORM OF TMS DERIVATIVES

RT[a] (methylene units)	Total ion current abundance		n^b	Metabolites
	Mean	Range		
10.61	24196	1046- 99792	10	Lactic acid, di-TMS
11.50	1318	238- 4665	8	3-Hydroxyisobutyric acid, di-TMS
12.17	553	145- 2282	6	3-Hydroxybutyric acid, di-TMS
12.49	1997	860- 8597	10	Urea, di-TMS
12 80	1058	244- 3959	10	Phosphoric acid, mono-TMS
13.18	2848	351- 4507	10	Succinic acid, di-TMS
14.86	1824	258- 4243	8	Malic acid, tri-TMS
15.23	656	228- 1856	6	Pyroglutamic acid, di-TMS
17.45	807	321- 1609	4	Aconitic acid, tri-TMS
18.35	1441	284- 3467	4	Hippuric acid, mono-TMS
18.94	1555	663- 2491	8	4-Hydroxyphenyllactic acid, tri-TMS
19.50	16923	868- 32325	10	Citric acid, tetra-TMS
20.15	7068	909- 13102	10	Palmitic acid, mono-TMS
21.03	416	266- 562	4	Heptadecanoic acid, mono-TMS
21.45	3157	599- 6315	7	Oleic acid, mono-TMS
22.07	9234	637- 12783	10	Stearic acid, mono-TMS
30.28	11343	5432- 18879	7	Cholesterol, mono-TMS

[a]Retention time in methylene units using a capillary column with 5% phenylmethylsilicone
[b]Number of occurrences in ten AF samples.

but its retention time and mass spectrum have been recorded, diagnosis can be based on the fingerprint principle, i.e., the change in the relative peak size. This approach allows the primary detection of unknown metabolic disorders.

Hence the screening of AF metabolites by means of high-performance capillary column GC–MS makes it possible to record at least 390 metabolites. In view of the individual variability of the content of AF metabolites, the normal metabolic profile must be represented by the total of the metabolites detected in all the normal samples stored in the computer memory. Each metabolite is characterized by its retention time, mass spectrum and the maximum ion current abundance of all the recorded identical metabolites. Such a 'maximum' metabolic profile can serve as a normal standard which the computer uses as a reference in diagnosing hereditary metabolic diseases.

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