Journal of Chromatography, 228 (1982) 43—50
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1149

CAPILLARY GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROFILES OF TRIMETHYLSILYL DERIVATIVES OF ORGANIC ACIDS FROM AMNIOTIC FLUIDS OF DIFFERENT GESTATIONAL AGE*

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(First received September 7th, 1981; revised manuscript received October 20th, 1981)

SUMMARY

Amniotic fluid from different gestational age patients was partitioned into neutral, acidic and basic fractions. The organic acids were trimethylsilylated and analyzed by glass capillary gas chromatography—mass spectrometry. A marked difference in the level of hippuric acid was observed between samples from early (15—22 weeks) and late (30—38.5 weeks) pregnancy. This difference probably reflects the degree of maturity in the fetal liver and kidney. The procedures establish amniotic fluid profiles of substances of varying gestational age and should be useful in determining alterations caused by diseases.

INTRODUCTION

Gas chromatographic—mass spectrometric (GC—MS) profiling of organic acids has been applied to various biological fluids, such as urine [1—5], serum [6], cerebral spinal fluid [7, 8], and amniotic fluid (AF) [9—14]. Multivariant analyses of biological specimens provide a more complete biochemical status of the individuals concerned. While many specific tests of AF have been used for prenatal diagnosis, such as alpha-fetoprotein [15] and acetylcholinesterase concentrations [16] for neural tube defects, chromosomal analysis for Down's syn-

*Taken in part from the Ph. D. Dissertation of K.J. Ng which was accepted at The Ohio State University, June 1981.

0378-4347/82/0000—0000/$02.75

drome, lecithin/sphingomyelin ratio [17] for fetal lung maturity and various enzymatic assays for various genetic/metabolic diseases, these tests do not present a broad chemical spectrum of the metabolic well-being of the fetus. Several GC—MS profiles of organic acids in amniotic fluid utilizing packed columns have been published [9—13] in which only normal amniotic fluid samples were analyzed for healthy mothers. We wish to report the use of a wall-coated open-tubular (WCOT) glass capillary column in the GC—MS analysis of human amniotic fluid from various gestational ages and certain problem pregnancies.

MATERIALS AND METHODS

Amniotic fluid samples

Twelve AF samples ranging from 15 to 38.5 weeks of gestation were obtained from patients seen at The Ohio State University Hospitals. Amniocenteses were necessitated for the assessment and management of fetal lung maturity in diabetic or Rh sensitized pregnancies, for prenatal screening of genetic diseases because of maternal age, for the detection of neural tube defects or other anomalies. The samples were frozen at −20°C until thawed for processing. Only glass syringes, carefully washed and sterilized were used to obtain specimens of AF. The clinical protocol used in this study was approved by our University Human Investigation Committee.

Reagents

The organic solvents used for extractions were of nanograde quality purchased from Mallinckrodt (St. Louis, MO, U.S.A.) or Omnisolv glass-distilled from MCB Manufacturing Chemists (Cincinnati, OH, U.S.A.). Hydrochloric acid solution, concentrated ammonium hydroxide, and saturated sodium borate buffer, which were used for adjusting pH and salting purposes, were extracted three times each with equal volumes of dichloromethane and ethyl acetate. Trimethylsilylating agent, N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.). Authentic organic acids were obtained from Sigma (St. Louis, MO, U.S.A.), Analabs (North Haven, CT, U.S.A.), and Applied Science (State College, PA, U.S.A.).

Extraction of amniotic fluid

Amniotic fluid samples in glass syringes were delivered immediately to the mass spectrometry laboratory and placed in a freezer at −20°C. In order to minimize enzymatic reactions during the thawing process, a 10-ml graduated cylinder was placed in ice while the syringe, with the needle removed, was supported above the graduated cylinder such that the thawed portion of the amniotic fluid flowed into the ice-cold graduated cylinder. An internal standard, phenyl-d_5-mandelic acid was added to each sample to obtain a concentration of 1.5 μg/ml. The sample was mixed and transferred to a pyrex glass tube and centrifuged at 10,000 *g* for 10 min. After centrifugation, the supernatant was gently poured into a separatory funnel with a glass stopcock and a glass stopper. To every 5 ml of amniotic fluid, 10 ml of pre-extracted saturated sodium borate buffer were added both for a salting effect and for adjusting the

pH to a value of 8.5—9.0. Twenty ml of dichloromethane were then added for every 5 ml of amniotic fluid. The separatory funnel was hand-shaken vigorously for 15 sec and the emulsion-like content was drained into pyrex tubes which were then centrifuged at 1400 *g* for 10 min. The upper aqueous layer was transferred with a pasteur pipette into the separatory funnel to be extracted two more times with dichloromethane. By means of a rotary evaporator connected to an aspirator, the organic portions containing neutrals and bases were pooled and reduced at 37°C to about 1 ml which was then transferred to a small vial (4-ml size, with a screw-cap lined with aluminum foil) and then blown dry with purified nitrogen at 35—40°C. The vial was stored to exclude moisture. The pH of the aqueous fraction was adjusted to 1.0 by adding 1 ml of 3 *N* hydrochloric acid. The acidified aqueous fraction was then extracted three times with 20 ml of dichloromethane. Each extraction was followed by centrifugation at 1400 *g* for 10 min at 25°C. The pooled dichloromethane extracts containing organic acids were dried as described above.

Derivatization of acidic fractions

One microliter of triethylamine and 30 μl of BSTFA were added to the dried acid extracts. The cap was immediately secured and the vial was vortexed for 15 sec. The sealed vial was then heated at 70°C for 35 min. The sample was allowed to cool prior to GC—MS analyses.

Gas chromatographic—mass spectrometric analysis of samples

Samples were analysed with a quadrupole mass spectrometer (Hewlett-Packard 5985 GC—MS system) equipped with a 5840A HP gas chromatograph and a 21MX E-series computer. A glass WCOT capillary column (25 m × 0.25 mm, coated with CP™ Sil 5, Chrompack, Middelburg, The Netherlands) was used, with helium as carrier gas at 4.3 ml/min and column head pressure at 0.68 bar (10 p.s.i.). The GC—MS analyses were performed under the following conditions. The temperatures of the injection port, the GC—MS interface and the ion source were set at 325°C, 350°C and 200°C, respectively. A splitless mode of injection was used with each sample size ranging from 0.2 to 3 μl depending on the concentration of organic acids in each sample. The temperature for GC analyses was programmed from 70°C, with a delay of 5 min, and then increased to 310°C at 8°C per min. The acquisition of mass spectral data was delayed for 3.5—4 min so as to vent off the solvent peak(s) and avoid contamination of the ion source. Mass spectral data were acquired utilizing electron impact (EI) at 70 eV and at an electron multiplier voltage of 3000 V.

RESULTS AND DISCUSSION

Utilizing the above procedures, capillary GC—MS total ion (TI) plots of trimethylsilylated organic acids from amniotic fluids were obtained (Figs. 1—4). The major components in each sample are labeled and minor peaks are numbered. A listing of all the components identified in the twelve amniotic fluid samples is presented in Table I. Components were identified by comparing the mass spectral data with those of authentic samples or with published spectra.

TABLE I

LIST OF IDENTIFIED COMPOUNDS WITH THE CORRESPONDING GC PEAK NUMBERS

Compound	Peak No.*	Compound	Peak No.
Lactic di-TMS	2	4-Acetylaminophenol di-TMS	40
2-OH-Butyric di-TMS	7	*p*-OH-Phenylacetic di—TMS	42
3-OH-Butyric di-TMS	8	Lauric TMS	44
3-OH-Isobutyric di-TMS	9	Homovanillic di-TMS	46
2-OH-2-Methylbutyric di-TMS	10	Hippuric di-TMS	48
2-OH-Isovaleric di-TMS	12	Hippuric TMS	49
Acetoacetic di-TMS	13	Myristic TMS	51
Benzoic TMS	14	Vanillylpropionic di-TMS	52
Urea di-TMS	15	Indole-3-acetic di-TMS	54
2-Ketoisocaproic di-TMS	17	Pentadecanoic TMS	56
2-OH-Caproic di-TMS	18	Palmitoleic TMS	58
2-Ketovaleric di-TMS	19	Palmitic TMS	60
2-Ketoisovaleric di-TMS	21	Indolepropionic di-TMS	62
Phenylacetic TMS	24	Heptadecanoic TMS	64
Phosphoric tri-TMS	25	Linoleic TMS	68
2-Keto-3-methylvaleric di-TMS	27	Oleic TMS	69
2-Ketocaproic di-TMS	28	Stearic TMS	70
Nonanoic TMS	29	Dioctyladipate	73
Decanoic TMS	30	Dioctylphthalate	74
Phenyl-d_5-mandelic di-TMS (I.S.)	32	Squalene	75
Phenyllactic di-TMS	38	Cholesterol TMS	80
p-OH-Benzoic di-TMS	39		

*Unused peak numbers are reserved for peaks yet to be identified.

Figs. 1 and 2 show the reproducibility of the profiles between individual patients, at 15.5 and 16 weeks of gestation, respectively. The profiles are generally characterized by an intense peak of lactic acid at the beginning and cholesterol at the end of the temperature-programmed run. Lactic acid is followed by a cluster of hydroxy- and keto-acids such as 2-hydroxybutyric, 3-hydroxybutyric, 3-hydroxyisobutyric, 2-ketoisocaproic, and 2-ketocaproic. Various long-chain fatty acids such as lauric, myristic, palmitic, stearic, oleic, palmitoleic, and linoleic and various aromatic acids such as phenyllactic, homovanillic, and hippuric, are distributed throughout the profiles. Phenyl-d_5-mandelic acid (at a concentration of 1.5 μg/ml; retention time of 15 min), the internal standard, was chosen because it eluted in a region free of other peaks. A deuterium-labeled phenyl-d_5 analogue of mandelic acid was used [18] to exclude confusion with endogenous mandelic acid.

Figs. 3 and 4 show typical profiles of organic acids in amniotic fluid of late gestation, 30 and 38.5 weeks, respectively. On examination of the profiles from various gestational ages, one outstanding and recurrent feature was that the amount of hippuric acid present in each amniotic fluid sample from pregnancies of late gestation (30—38.5 weeks) was greater than that in early pregnancy (15—20 weeks) as determined by the ratios of the peak area of hippuric acid to that of the mandelic acid, the internal standard. In fact the mean of eleven samples reveals a six-fold increase in hippuric acid between early and late gestation. Fig. 5 shows a plot of the ratios of the peak areas of hippuric acid to the peak areas of the internal standard versus the gestational age. The plot clearly re-

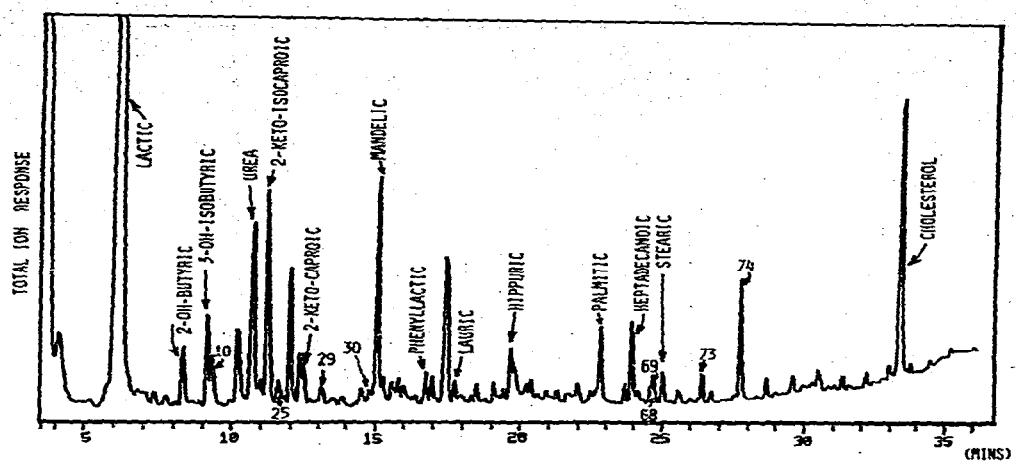

Fig. 1. Total ion plot of a trimethylsilylated dichloromethane extract of the acid components from an amniotic fluid from a 15.5-weeks pregnancy; the specimen was drawn for karyotyping. d_5-Mandelic acid (retention time 15 min) and heptadecanoic acid (23.5 min) were added as internal standards (each at 1.5 μg/ml). GC—MS conditions are described in the text. The numbered peaks are as explained in Table I.

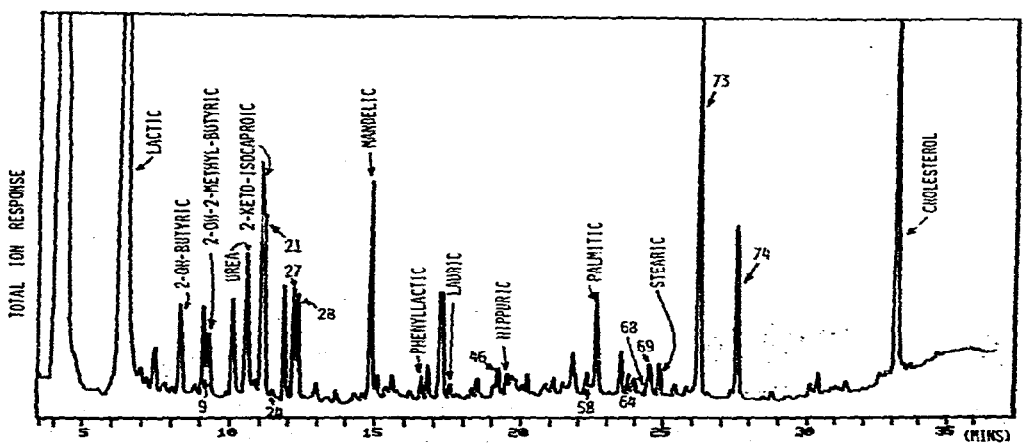

Fig. 2. Total ion plot of a trimethylsilylated dichloromethane extract of the acid components from an amniotic fluid from a 16-weeks pregnancy; the specimen was drawn for karyotyping.

flects an increase of hippuric acid with gestational age.

Hippuric acid is mainly synthesized in the liver in man [19]. The formation of hippuric acid depends on the availability of benzoic acid, glycine and various enzymes such as activating enzyme and glycine N-acyltransferase [20]. Whether the observed increase of hippuric acid in amniotic fluid with gestational age is of maternal or of fetal origin is yet unknown. If it were of maternal origin, the increase could be explained by assuming (a) possible changes with the duration of gestation of the maternal liver in the production and/or of changes of maternal kidneys in the elimination of hippuric acid and (b) increasing transfer placentally or otherwise of hippuric acid with gestational age from the maternal to the fetal compartment. If the benzoic acid content of the

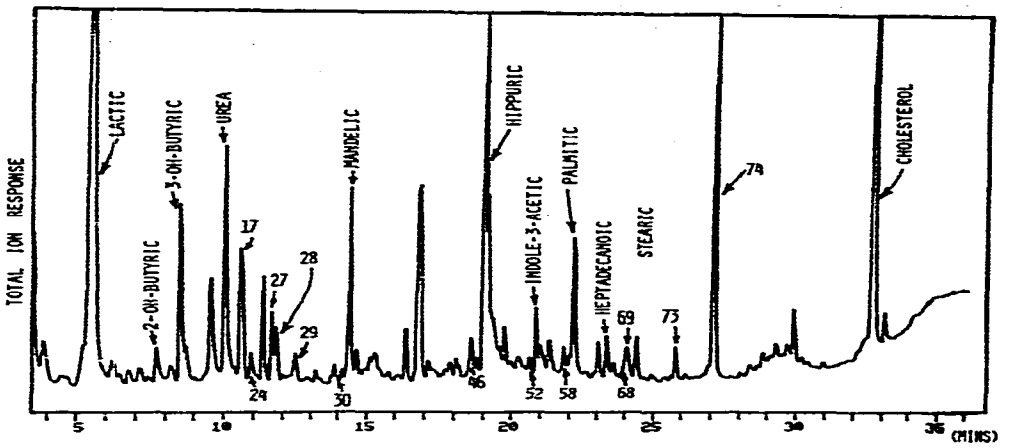

Fig. 3. Total ion plot of a trimethylsilylated dichloromethane extract of the acid components from an amniotic fluid from a 30-weeks pregnancy; the specimen was drawn because of severe Rh-immunization.

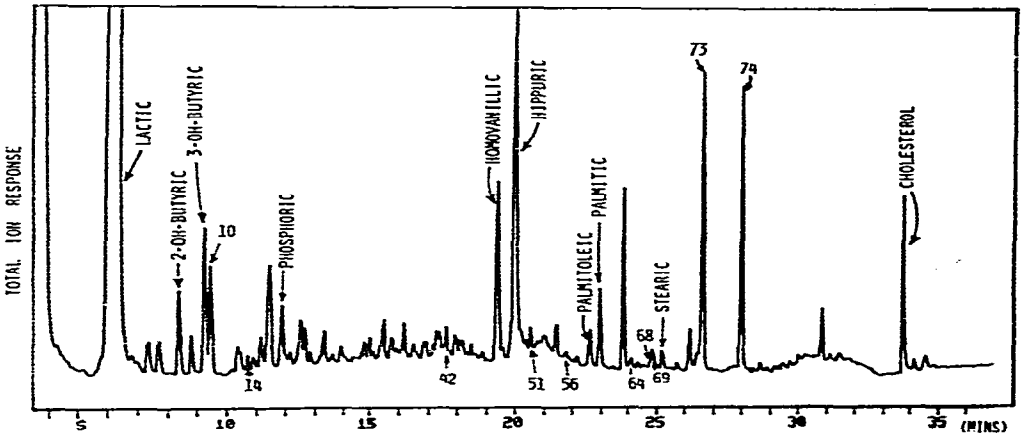

Fig. 4. Total ion plot of a trimethylsilylated dichloromethane extract of the acid components of amniotic fluid from a 38.5-weeks pregnancy; the specimen was drawn for lecithin/sphingomyelin ratio determination.

diet of the mother were responsible for the observed difference, one would find random fluctuation in the level of hippuric acid in amniotic fluid from different patients, or one would have to assume that our sampled pregnant women somehow consumed more benzoic acid in their diet as pregnancy progressed. If on the other hand, the observed increase of hippuric acid in amniotic fluid were of fetal origin, the increase may represent the increasing maturity of the enzyme function of fetal liver to produce hippuric acid by the condensation of benzoyl thioester of coenzyme A with glycine, as well as the maturity of the fetal kidneys in the excretion of hippuric acid. It is known that the constituents of amniotic fluid change quantitatively and qualitatively with gestation, resembling those of maternal serum in early pregnancy and those of fetal urine in late pregnancy. It has been reported that the amount of hippuric acid excreted in urine in neonates increased with age [21], indicating the maturity of

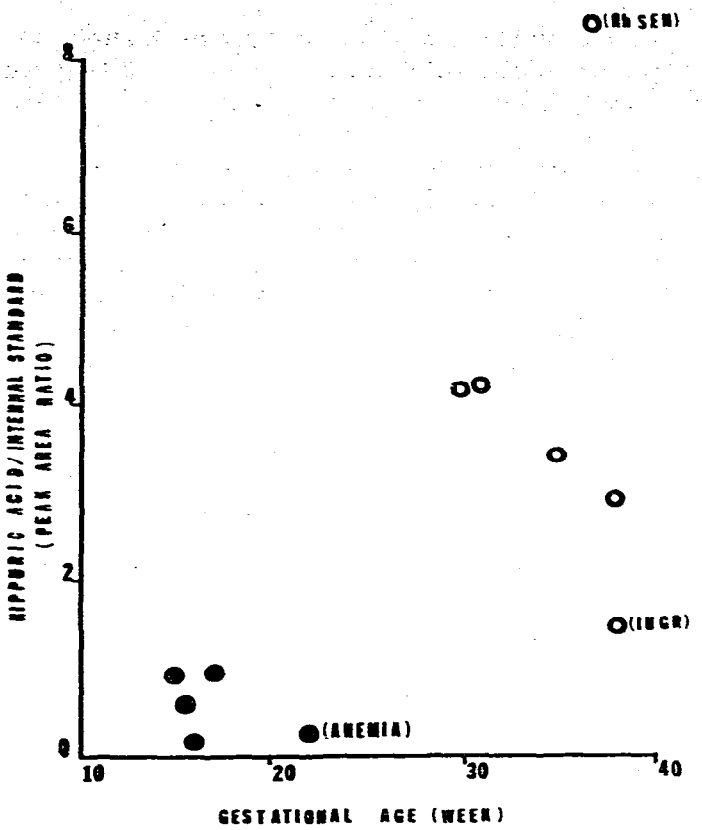

Fig. 5. Plot of the ratios of the peak areas of the internal standard to those of hippuric acid (measured from the total ion plots) versus the gestational age. d_5-Mandelic acid was added as an internal standard at 1.5 μg/ml to 11 of the 12 amniotic fluid samples. Two abnormally low ratios, one suffering from malnutrition and anemia (at 22 weeks pregnancy) and the other diagnosed as intrauterine growth retardation (IUGR) (at 38 weeks pregnancy), and one unusually high ratio, the patient being Rh sensitized (at 37 weeks pregnancy).

the glycine conjugating pathway to eliminate aromatic acids. The profiling procedures presented here clearly demonstrate that hippuric acid can be followed throughout gestation as a monitor of fetal liver and kidney function.

Various other methods for profiling organic acids in biological fluids have been reviewed [22, 23]. The use of anion-exchange methods, or adsorption of organic acids to silica gel followed by elution with a suitable solvent system may give better quantitative recovery. However, these procedures would preclude the simultaneous analysis of neutral and basic components with the same sample.

A comparison of the organic acids identified in amniotic fluid to the recent literature findings [9—13] shows that our extraction procedure recovered less of the polar compounds (e.g. citric, isocitric, tetronic, 2-hydroxyglutaric, glyceric acids) while other components which have not been reported from AF can be identified by our procedures. These components include indole-3-acetic, indole-3-propionic, vanillylpropionic, 2-hydroxy-2-methylbutyric, 2-hydroxyisovaleric, acetoacetic, benzoic, 2-hydroxycaproic, 2-ketovaleric,

phenylacetic, nonanoic, decanoic, phenyllactic acids, and 4-aminophenol. The isolation of new compounds from amniotic fluid can be partly accounted for by the use of a different extraction solvent, and the exclusion of deproteination before the extraction procedures.

On examination of capillary GC—MS profiles of organic acids from pregnancies of various gestational age an obvious increase of hippuric acid with gestational age was observed. In addition, indole-3-acetic acid was either absent or present in a relatively very low level in most samples. However, in samples associated with open neural tube defects, Rh sensitized pregnancies and a sample from a pregnant woman who smoked, the levels of indole-3-acetic acid were much higher. We are currently investigating the significance of this finding.

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