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GAS PHASE ANALYTICAL METHODS FOR THE STUDY OF HUMAN METABOLITES

METABOLIC PROFILES OBTAINED BY OPEN TUBULAR CAPILLARY CHROMATOGRAPHY

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SUMMARY

A method has been developed for the preparation of high-resolution (100,000 or more theoretical plates), thermostable glass open tubular capillary columns. The key to column preparation lies in the inclusion of silanized silica particles (Silanox, 6–10 μ diameter) in the film. A special coating process is used for SE-30 columns. These columns have been used in metabolic profile separations of human urinary steroids, urinary sugars and polyols, and drugs and drug metabolites.

INTRODUCTION

Multicomponent analyses of very small samples of complex mixtures of biologic origin can be carried out by gas chromatographic methods. This capability has been used to obtain metabolic profiles based upon a variety of groups of compounds present in human body fluids. Methods have been developed for carrying out separations with high-resolution, thermostable, open tubular glass capillary columns with theoretical plate efficiencies of 100,000 or higher. Very few instances of peak overlap occur under these conditions.

Metabolic profiles can be used for different purposes. The avenues now under exploration are: (a) defining "metabolic normality" insofar as this can be done with a limited number of examples, (b) defining the association between non-normal profiles and pathologic states, (c) describing developmental changes, and (d) defining metabolic pathways for exogenous substances, including drugs and environmental toxic agents.

The studies described here involve urinary steroids, urinary sugars and urinary drug metabolites.

EXPERIMENTAL

Preparation of open tubular glass capillary columns

Procedures used in the preparation of open tubular glass capillary columns are described in detail in earlier papers[1,2]. In current work, columns of 60 m length, 1.0 mm O.D. and 0.3 mm I.D., in coils approximately 12 cm in diameter, are employed. These are made with a glass drawing and coiling apparatus (Hupe and Busch, Groetzingen/Karlsruhe, G.F.R.), and are silanized (with 10% v/v dimethyldichlorosilane in toluene) before coating.

The significant material added to create a special form of film is Silanox (Grade 101; Cabot, Boston, Mass., U.S.A.). This is a silanized silica powder, 6–10 μ in diameter, which is hydrophobic, does not self-aggregate and which shows no adsorptive behavior when added to liquid phases. It is introduced by coating columns with a suspension of Silanox in a solvent of high density (chloroform or carbon tetrachloride) containing the liquid phase. The best procedure for preparing SE-30 columns is to use a two-step coating process. The initial coating, containing Silanox, is attained by using a dynamic coating process in which a suspension of Silanox (2 g/100 ml) in carbon tetrachloride containing SE-30 (0.5 g/100 ml) is propelled through the column. After drying, a second coat of SE-30 is applied through use of a 2% solution of SE-30 in isooctane.

Additional experience in the use of this procedure has indicated that there is only one problem which may occur in the coating process. The flow may be blocked if the suspension of Silanox is not fully dispersed (a short sonication period is advisable before the suspension is used), or if a plug of solvent is not employed to wet the column before the suspension is introduced. When appropriate precautions are taken, no difficulties are encountered.

Columns prepared in this way show approximately 100,000 theoretical plate efficiency for tetracosane at 250° (HETP 0.60 mm); the highest value observed for a 60-m column was 150,000 theoretical plates (HETP 0.40 mm).

Inlet system

The inlet system has been described[3]. A short (5-cm) packed column was used to achieve complete vaporization before splitting. A 1-cm section of 10% SE-30 on acid-washed and silanized Gas-Chrom P was used at the head of the column; this is an important provision to protect certain types of methoximetrimethylsilyl ether derivatives from structural alteration[4]. The inlet gas flow was flow controlled (5–10 ml/min), while the capillary gas flow (1–2 ml/min) was pressure controlled. The usual split ratio was 5:1 to 10:1; the carrier gas was helium. The precolumn was replaced at intervals of about two weeks when biologic samples were under study.

Gas chromatographs

A Barber-Colman Model 5077 and an F and M Model 400 gas chromatograph (F and M Division, Hewlett-Packard, Avondale, Pa., U.S.A.) were modified to accept capillary columns and the inlet system. Keithley Model 417 picoammeters were used; the usual full-scale response was 3×10^{-11} A.

Human urinary steroids

Analytical samples of human urinary steroids (24-h collection) were converted to trimethylsilyl (TMS) and methoximetrimethylsilyl (MO-TMS) derivatives by the method of Thenot and Horning[5].

Human urinary aldoses and alditols

Analytical samples of human urinary aldoses and alditols (24-h collection) were converted to acetylaldononitriles and acetylalditols by the method of Szafranek *et al.*[6].

Human drug metabolic profiles

Human urinary (24-h collection) drug metabolites were isolated and derivatized following the procedure of Horning *et al.*[7].

RESULTS AND DISCUSSION

The high-resolution separation of components of mixtures of biologic origin, through use of thermostable open tubular glass capillary columns, has been for many years an objective of laboratories engaged in gas phase analytical studies in medical and biologic research work. The observation by Golay[8] in 1958 that an open tubular capillary column could be used for gas–liquid chromatographic separations led to many attempts to prepare glass columns suitable for general use; the experience of every investigator, however, was that liquid phases on glass formed micro-droplets when heated and cooled[9]. Consequently, almost all work with biologic samples has been carried out with packed columns. Thin-film (1 % of liquid phase) columns were developed for the separation of steroids in 1960[10], and most later work with thermostable packed columns was based upon the procedure of Horning *et al.*[11] for the preparation of column packings.

The fact that the best packed columns were made with supports of the diatomaceous earth type, while glass open tubular columns were unsatisfactory because of film break-up on heating[9] and because not as much phase as desired could be deposited on a smooth surface, led to the view that wall modification was a necessity for the preparation of glass open tubular columns. The best known columns of this type are those of Grob[12]; the liquid phase is applied after surface modification. A surface etching procedure was also described by Novotny and Tesarik[13]. An evaluation of the usefulness of Grob columns in steroid separations was carried out by Völlmin and his colleagues[14–16] and by Ros and Sommerville[17]; Novotny–Tesarik columns were evaluated by Novotny and Zlatkis[18]. A different method of altering the wall, with the creation of surface microirregularities, is to introduce particulate material which will adhere to the glass surface. Golay[19] used colloidal clay, and many other materials have been evaluated for possible use in this way. This concept is not greatly different from that of wall etching, in that the aim was to create a surface with microirregularities (considered to be necessary to prevent film break-up) which would accept more liquid phase than a smooth surface. Support-coated open tubular (SCOT) columns are examples of columns prepared in this way.

The glass open tubular capillary columns used in this work have proved to

be thermostable and to have long lifetimes if not destroyed by accident. The concept involved in their preparation is different from that employed earlier. Column stability is due to the presence of small particles of silanized silica (Silanox 101); this material does not self-aggregate or adhere to the glass wall. The glass surface is silanized to reduce adsorption, but it is not etched or otherwise treated. A smooth surface is believed to be desirable in order to obtain a uniform film. In the preparation of SE-30 columns, it is best to use a two-step process (since the viscosity of a suspension of Silanox in a solution containing SE-30 is high). The initial coating serves to introduce the Silanox into the column, while the second introduces additional liquid phase. The coating method is not greatly different from that described by Schwartz and his colleagues[20,21] for the preparation of open tubular capillary adsorption columns. The Schwartz columns, which were prepared using a commercial colloidal sol of hydrophobic silica, gave excellent results in hydrocarbon separations[21]. Although these steel columns contained no liquid phase, it was noted that a liquid phase could be added during the coating process if desired. The purpose of the work, however, was to prepare gas–solid adsorption columns.

Metal open tubular capillary columns have found many applications in hydrocarbon chemistry, and in work with compounds of relatively low molecular weight. The work of Teranishi, for example, in developing metal capillary columns for studies of flavors and odors is well known. Attempts to use metal columns at elevated temperatures (200–300°) in analyses of biologic samples have never been highly successful. In theory, a smooth and inert surface is all that is required for the method described here, suggesting that some metals could be used. At this time the preferred material is glass.

Recent studies indicate that there are also other ways of preparing capillary columns. Rutten and Luyten[22] described a method involving pretreatment of glass capillaries with surface-active agents, followed by coating with a nonpolar phase. Blumer[23] described a coating method in which Silanox was added to a solution of a liquid phase, followed by coating by a dynamic process. We are currently studying the problem of preparing polar open tubular glass capillary columns comparable in stability to the SE-30 columns used in this work.

A typical alkane separation is shown in Fig. 1; a 30-m column was used under isothermal conditions. Very little trailing was observed; the column efficiency was good, but not adequate for current biologic studies. The compelling reason for seeking high-resolution columns in biologic work is illustrated in Fig. 2. A biologic sample containing urinary drug metabolites from N,2-dimethyl-2-phenylsuccinimide (an antiepileptic drug) was derivatized, and an analytical separation was carried out with a packed column (P, 5,000 theoretical plates) and a capillary column (C, 100,000 theoretical plates). Two regions of the separation charts are shown in the figure. One peak, of essentially theoretical shape (packed column), contained two components when examined by capillary chromatography. A second peak, apparently containing two components, was found to contain four components when capillary separation conditions were used.

Another useful property of columns prepared with Silanox is their ability to accept relatively large samples of complex mixtures containing components present in widely different amounts. Fig. 3 shows a separation of human urinary steroids as trimethylsilyl (TMS) and methoximetrimethylsilyl (MO-TMS) derivatives. The major

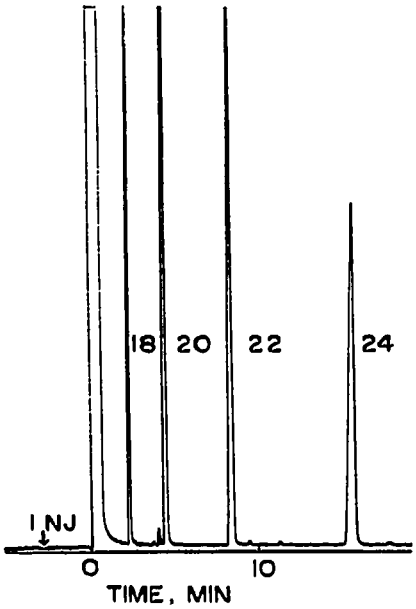

Fig. 1. Isothermal separation of *n*-alkanes with a 30 m × 0.3 mm I.D. glass capillary column (SE-30 phase) at 225°. The injection system permits the use of the usual type of solutions (1- to 5-μl samples). The full-scale chart response usually corresponds to 3×10^{-11} A.

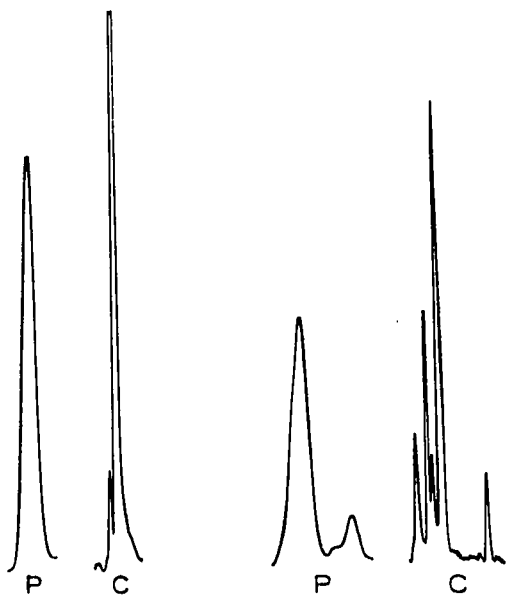

Fig. 2. Comparison of separations for a biologic sample containing metabolites of methsuximide. The "P" peaks are from a packed column separation (SE-30, 5000 theoretical plates); the "C" peaks are the same peaks in a capillary column separation (SE-30, 100,000 theoretical plates).

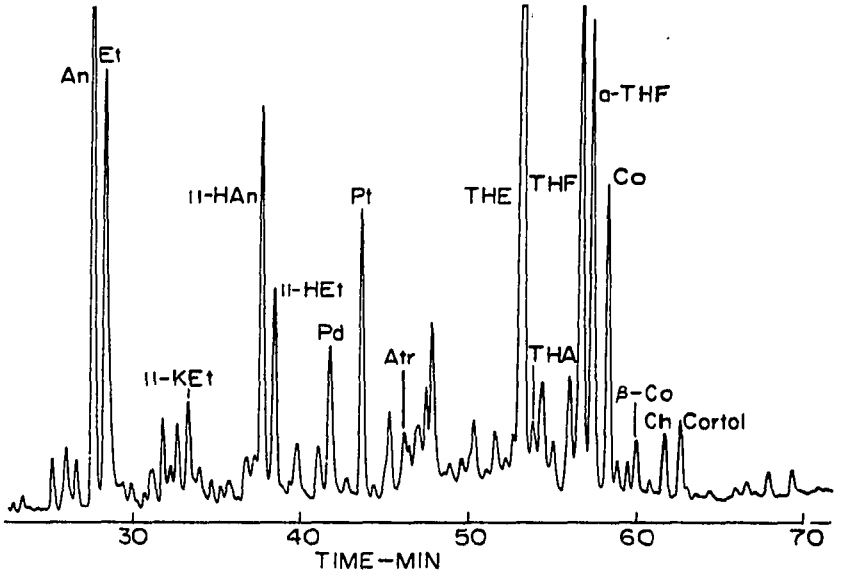

Fig. 3. Separation of human urinary steroids as TMS and MO-TMS derivatives with a 30 m × 0.3 mm I.D. glass capillary column (SE-30, temperature programmed at 1°/min from 200°). This is a typical adult male profile. The compounds are: An = androsterone; Et = etiocholanolone; 11-KEt = 11-ketoetiocholanolone; 11-HAn = 11-hydroxyandrosterone; 11-HEt = 11-hydroxyetiocholanolone; Pd = pregnanediol; Pt = pregnanetriol; Atr = androst-5-ene-3β,16α,17β-triol; THE = tetrahydrocortisone; THA = 5β-pregnane-3α,21-diol-11,20-dione; THF = tetrahydrocortisol; α-THF = *allo*-tetrahydrocortisol; Co = cortolone; β-Co = β-cortolone; Ch = cholesterol; cortol.

constituents of the sample appear as off-chart peaks, but there is no evidence of column overload, and many minor constituents are separated.

Fig. 3 shows a metabolic profile of the urinary steroids of a normal adult male and Fig. 4 shows a corresponding profile of urinary steroids for a normal adult

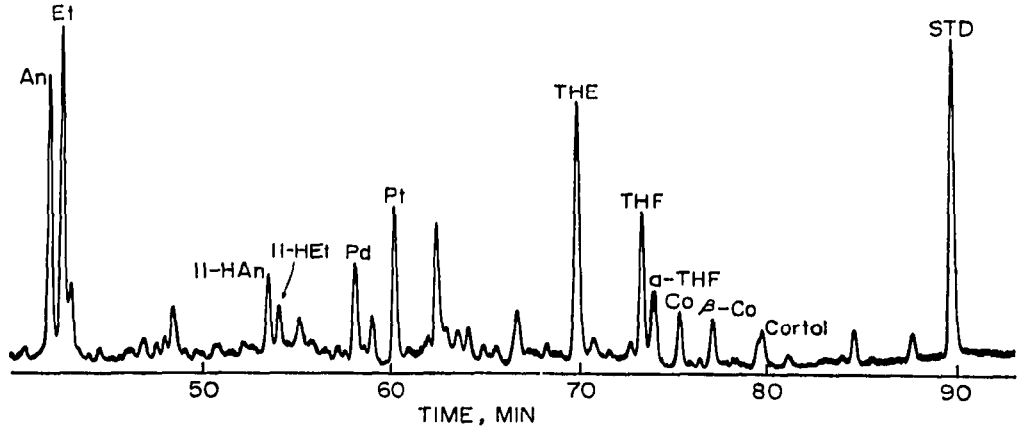

Fig. 4. Separation of human urinary steroids as TMS and MO-TMS derivatives with a 60 m × 0.3 mm I.D. glass capillary column (SE-30, temperature programmed at 1°/min from 220°). This is an adult female profile. STD = Standard. The compounds are the same as in Fig. 3.

female. While comparatively few of these high-resolution profiles are available for study, several interesting observations have been made. The reduction of 3-one-4-ene to 3-ol steroids is carried out in the liver in a way that appears to be associated with sex characteristics. The ratio of androsterone to etiocholanolone (An:Et) is about 2:1 for adult males, and the ratio of tetrahydrocortisol to *allo*-tetrahydrocortisol (THF:α-THF) is about 1:1. For adult females, the An:Et ratio ranges from about 1:2 to 1:1, while the THF:α-THF ratio is about 3:1 to 3:2. No exceptions have been found to the observation that the 5α to 5β ratio of reduction products varies in parallel for the An:Et pair and the THF:α-THF pair; this suggests that a single enzyme is involved, or that similar control mechanisms are acting on related enzymes.

Preliminary observations have been made with respect to the association of non-normal profiles with pathologic circumstances. Figs. 5 and 6 show steroid metabolic profiles for two patients with polycystic ovaries. It is known that these patients frequently show an elevated excretion of 17-ketosteroids (Fig. 5 shows this effect), but it is less well known that the pattern of $5\alpha/5\beta$ reduction is typically male. This is clearly evident in both figures.

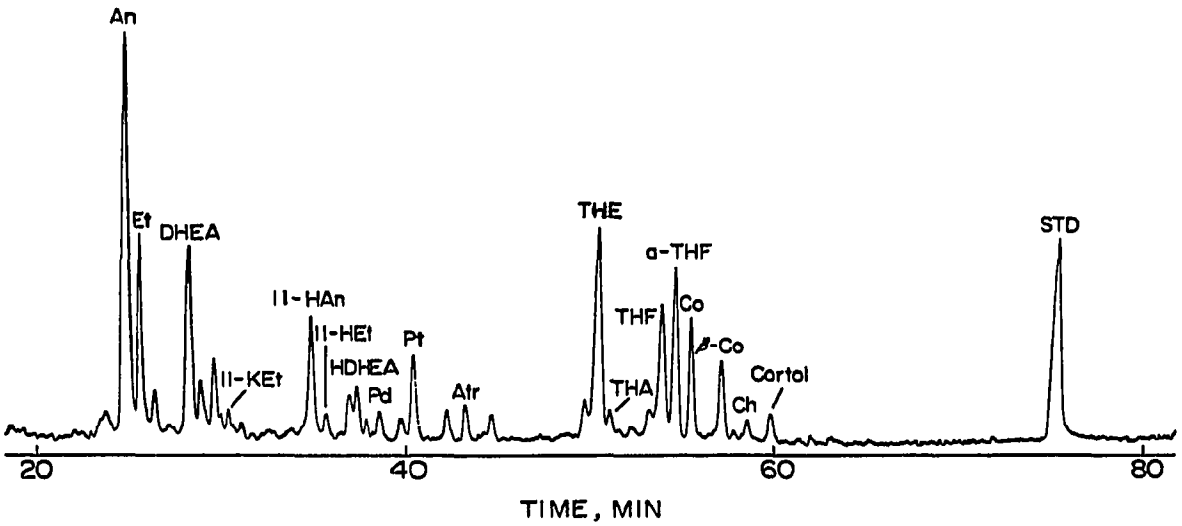

Fig. 5. Separation of human urinary steroids as TMS and MO-TMS derivatives with a 30 m × 0.3 mm I.D. glass capillary column (SE-30, temperature programmed at 1°/min from 200°). This is a profile for a patient with polycystic ovaries. STD = Standard; DHEA = dehydro epiandrosterone; HDHEA = 16α- hydroxy dehydro epiandrosterone. The other compounds are the same as in Fig. 3.

The generalization that adult males and females are not identical in all metabolic pathways is well established; at the same time, the basis of these differences may not be the obvious one of hormonal control by secreted steroids. For example, the association of a pathologic state for females with a male reduction enzyme pattern suggests, as a first explanation, that in this instance the ovaries are secreting androgens in excess amount, and that the liver is acting under direct androgenic hormonal stimulus. In other words, these associative data can be interpreted in

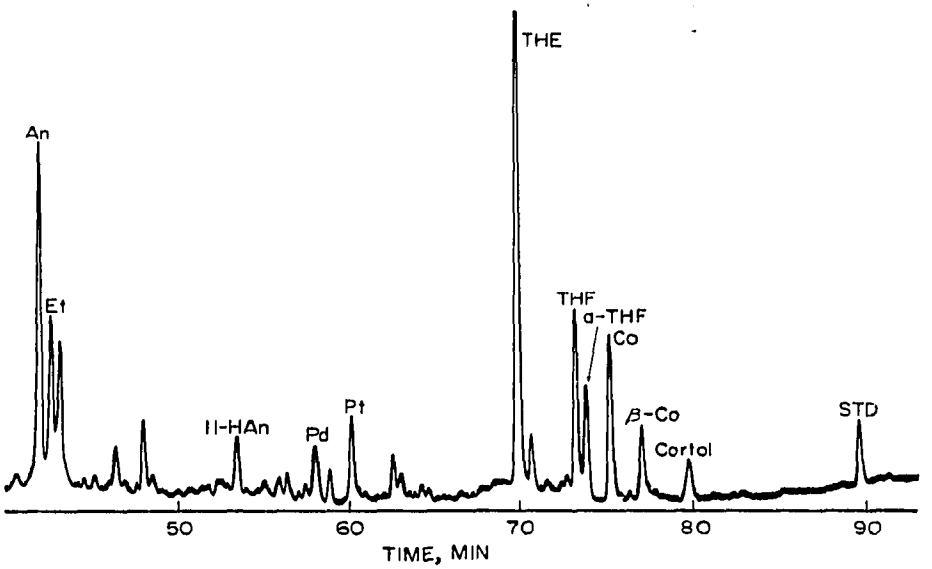

Fig. 6. Separation of human urinary steroids as TMS and MO-TMS derivatives with a 60 m × 0.3 mm I.D. glass capillary column (SE-30, temperature programmed at 1°/min from 220°). This is also a profile for a patient with polycystic ovaries. STD = Standard. The compounds are the same as in Fig. 3.

cause–effect terms, with the physiologic change representing the cause and the reduction ratio changes representing the effect. There are indications, however, that this explanation may not be correct. In a study of steroid reductase pathways in rat liver, Maume *et al.*[24] found definitive evidence of sex-associated reductases. This basic effect, recognized first by Forchielli *et al.*[25], in 1958, may therefore be a general one. If it can be shown, however, through the tissue culture work of Padieu[24] and his colleagues that this cellular behavior is maintained in the absence of steroids other than corticosteroid substrates, the conclusion which is indicated is that the pattern of steroid reductases in the liver is a consequence of a developmental process. The association between a male steroid reductase pattern in the liver and excessive androgen production may be due to a common cause: that of an androgenic effect imposed during an early developmental period upon two different organs in the female. The logical extension of this interpretation is the view that the observed physiologic change in the ovaries may occur in the adult as a consequence of an event occurring during an early period of organ development.

Metabolic profiles may also be used in studies of developmental changes occurring during the first few days or weeks of life. Fig. 7 shows a profile of urinary steroids, as TMS and benzyloximetrimethylsilyl (BO-TMS) derivatives, for a newborn infant. Most of the compounds are 3β-ol-5-ene steroids which originate in cells of the fetal adrenal zone; these gradually disappear and are replaced by cells characteristic of adult adrenals.

Figs. 8 and 9 show urinary aldose and polyol profiles for a newborn male infant and for the mother. Both profiles are normal, but they are different. Myoinositol is the major component of this group of urinary compounds for the newborn.

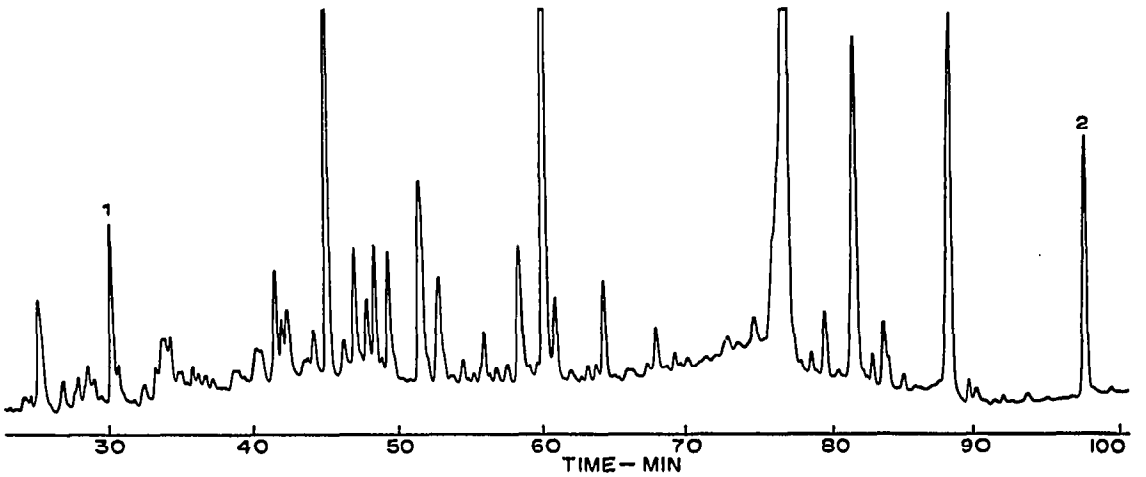

Fig. 7. Human urinary steroid profile of a newborn infant. The steroids were separated as TMS and BO-TMS derivatives with a 30 m × 0.3 mm I.D. glass capillary column (SE-30, temperature programmed at 1°/min from 200°). Most of these steroids have a 3β-ol-5-ene structure. 1 = C_{26}; 2 = C_{38}.

This may be due to the fact that the central nervous system is still undergoing development at this time. Very little myoinositol is excreted by adults. Most of the identified compounds in both profiles are alditols.

Certain types of pathologic circumstances lead to major changes in these

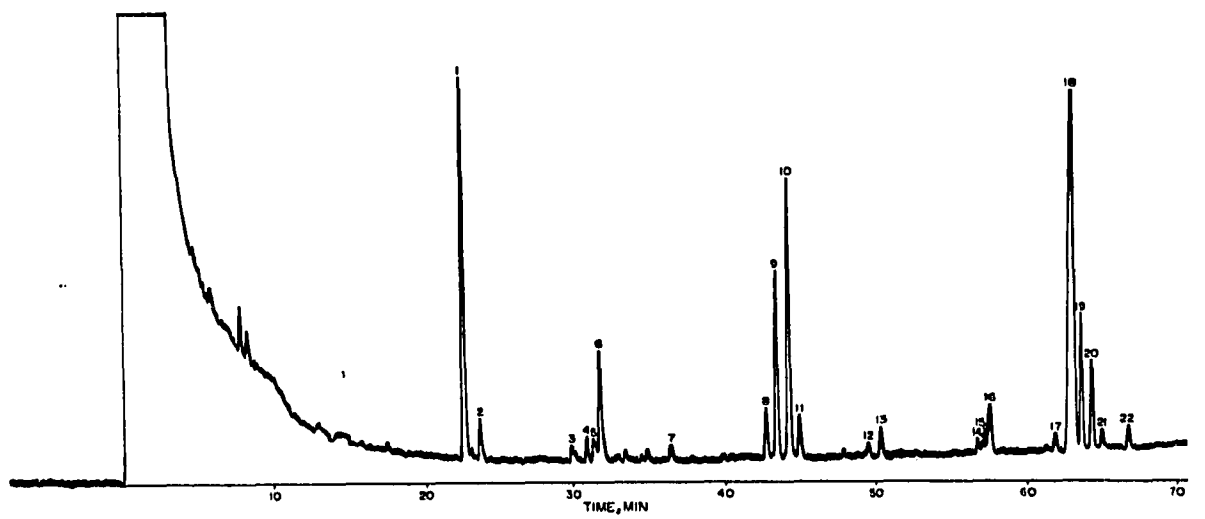

Fig. 8. Profile of human urinary polyols and sugars of a newborn male infant. The compounds were separated as acetyl and acetylaldononitrile (Wohl reaction) derivatives. The column was a 60 m × 0.3 mm I.D. glass capillary (SE-30 phase); the separation was temperature programmed at 1°/min from 150°. The compounds which have been identified are: 1 = erythritol; 2 = threitol; 8 = ribitol; 9 = arabinitol; 11 = xylitol; 13 = glucose; 18 = myoinositol; 19 = mannitol; 20 = glucitol; 21 = galactitol.

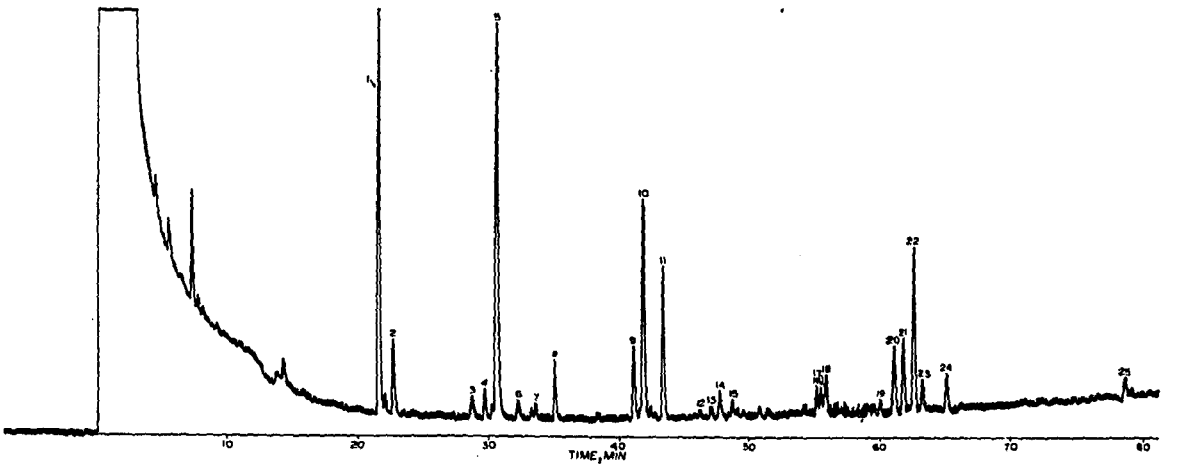

Fig. 9. Profile of human urinary polyols and sugars of the mother of the infant of Fig. 8; the methods are those used for Fig. 8. A single urinary collection was employed. The compounds which have been identified are: 1 = erythritol; 2 = threitol; 9 = ribitol; 10 = arabinitol; 11 = xylitol; 15 = glucose; 20 = myoinositol; 21 = mannitol; 22 = glucitol; 23 = galactitol.

profiles. Adult diabetics show varying amounts of glucose (the analytical procedure requires modification when large amounts of glucose are present), and galactosemia would lead to a greatly altered profile.

Fig. 10 shows a separation of drugs and drug metabolites. Most drugs form a number of metabolites; characteristic reactions include aliphatic chain hydroxylation, aromatic epoxide formation followed by dihydrodiol formation, or conversion

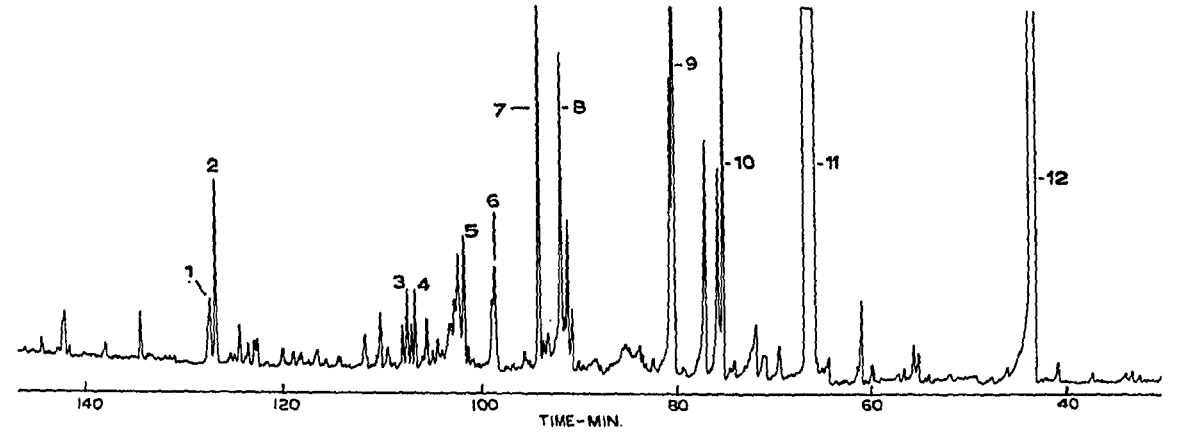

Fig. 10. Profile obtained with a 60 m × 0.3 mm I.D. glass capillary column (SE-30 phase) of methyl and methyltrimethylsilyl derivatives of drugs and urinary drug metabolites from a human receiving dilantin, phenobarbital and methsuximide. The compounds that have been identified include the parent drugs and a variety of metabolites including dihydrodiols formed through the epoxide–diol pathway. The order of elution is from right to left in this chart. 1 = Dihydrodiol derivative of dilantin; 2 = hydroxydilantin; 3, 4 = dihydrodiol derivatives of hydroxymethsuximide; 5 = dilantin; 6 = *p*-hydroxyphenobarbital; 7 = diol derivative of methsuximide; 8 = dihydrodiol derivative of methsuximide; 9 = *p*-hydroxymethsuximide; 10 = C_{16}; 11 = phenobarbital; 12 = methsuximide.

to phenols, epoxidation and diol formation from unsaturated carbon–carbon bonds, demethylation, oxidation of alcohols to ketones as well as the reverse reaction, and a variety of conjugation reactions. The relative amount of each product in a multiproduct reaction scheme is determined by relative rates; these rates in turn vary with each species and with individual considerations which include age, sex, genetic background, nutritional state, and extent of drug–drug interaction. Gas phase analytical methods, because of their capability of providing multicomponent analyses, are highly valuable in studies of drug metabolism. They are also useful in toxicologic studies. The advantage of capillary column chromatography over packed column chromatography is illustrated in Fig. 2.

CONCLUSION

High-resolution (100,000 or more theoretical plates), thermostable, open tubular glass capillary columns may be prepared by a new process which involves the incorporation of silanized silica particles (Silanox) in a special type of film on the wall. These small particles do not self-aggregate or adhere to the wall, but their presence leads to highly thermostable columns.

These columns are particularly useful in studies involving complex mixtures of biologic origin. They can be used in metabolic profile work involving the characterization of normal and pathologic states, the study of developmental changes, and the characterization of multiproduct foreign substance metabolism.

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