Metabolic Profiles: Gas-Phase Methods for Analysis of Metabolites

E. C. Horning and M. G. Horning

Multicomponent analyses were carried out for three types of urinary constituents: steroids, acids, and drugs and drug metabolites. The methods were based on gas-phase analytical techniques, which include the use of instruments and instrumental systems for gas chromatography, gas chromatography–mass spectrometry, and mass spectrometry–computerization. After isolating an analytical sample, we prepared derivatives in each instance. Gas chromatography was used for separations, mass spectrometry for identification. These procedures for obtaining metabolic profiles may be used in various ways, including studies of abnormal conditions, drug metabolism, and the effects of drugs on metabolic pathways, as well as for human developmental studies.

Additional Keyphrases *normal and disturbed carbohydrate metabolism • organic acids in urine • gas chromatography • mass spectrometry • computers • drug metabolism • metabolism of newborns and infants • steroids*

Gas-phase analytical methods are currently based on the use of GC[1] techniques for separation purposes, MS methods for identification and establishing structure, and COM for conversion of instrumental data into qualitative and quantitative chemical information. MS has been used in special applications for discrimination purposes, without a separation step, but for the study of metabolic profiles it is necessary to use sequential procedures, which may be combined in a single instrumental system. The current state of instrumental development is represented by GC–MS–COM systems.

These methods may be used to detect and estimate a single substance, or many in one analysis. In essence, "metabolic profiles" are multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites. Each component of interest can be identified by MS methods.

Here, we describe metabolic profile procedures developed for study of urinary steroids, urinary acids, and neutral and acidic urinary drug metabolites. Other profile procedures that have been developed in this laboratory include those for sugars and related polyols (alditols) and acids of the Krebs cycle and related compounds. Still others are under development. Profiles may prove to be useful for characterizing both normal and pathologic states, for studies of drug metabolism and the effects of drugs on human metabolism, and for human developmental studies.

Materials and Methods

GC

Model 5000 (Barber-Colman Co., Rockford, Ill. 61101) instruments with Model 417 picoammeters (Keithley Instruments, Inc., Cleveland, Ohio 44106) and Texas Instrument (Houston, Texas 77006) recorders were used. The columns were 365 cm × 4 mm glass W-tubes; column packings were 1% SE-30, 5% SE-30, and 1% Dexsil on 100–120 mesh Gas Chrom P, prepared according to our usual method (*1*). The conditions were: injector zone, 250–260°C; detector bath (hydrogen flame ionization), 300°C; nitrogen flow rate, 30–40 ml/min (column at 200°C); temperature programming, usually 1° or 2°C/min.

Baylor College of Medicine, Houston, Tex. 77025.

[1] Nonstandard abbreviations used: GC, gas chromatography (-ic); MS, mass spectrometry(-ic); COM, computer; BO, benzyloxime; TMSi, trimethylsilyl-; ME, methyl ester or methyl derivative; BSTFA, bis-trimethylsilyltrifluoracetamide; MO, methoxime; amu, atomic mass unit(s).

GC–MS

A Model 9000 instrument (LKB Instruments, Inc., Rockville, Md. 20852) was used, with a 270 cm × 4 mm glass coil column. Ionizing current was 60 μA; voltage was 70 eV; the ion source was at 250°C; and scan time was 3–6 s.

GC–MS–COM

This system consisted of a Model 1015 quadrupole mass spectrometer (Finnigan Instruments Corp., Sunnyvale, Calif. 94086), with a chemical ionization source, and a modified GC unit (Varian Aerograph, Palo Alto, Calif. 94303), together with a computer system based upon a PDP 8/I computer (System Industries, Sunnyvale, Calif. 94086) and using a Houston Complot plotter (Houston Instrument, Bellaire, Texas 77041). The GC column was 185 cm × 1 mm (i.d.) glass coil; separations were temperature programmed at 4–6°/min, with methane as the carrier gas. The MS source was operated with 0.5–1 Torr pressure of methane, with voltage of 80 eV and scan time 2-3 s in repetitive mode.

Samples

Sample isolation. Urinary steroids from adults and newborns were isolated according to a commonly used procedure (*2*) involving enzymic hydrolysis with "Glusulase" (Endo Laboratories, Inc., Garden City, N. Y. 11530) and solvent extraction. The Sephadex separation was omitted. Urinary acid fractions were obtained according to a commonly used procedure (*2*). Urinary drug metabolites were obtained by a procedure modified from that developed for urinary acids. The effluent from a DEAE-Sephadex column (*2*), containing neutral and basic constituents, was extracted with ether and ethyl acetate. The combined organic extract was dried (magnesium sulfate) and the solvents were evaporated (rotary evaporator). The residue was dissolved in 1 ml of methanol.

Derivative formation. Steroid benzyloxime-trimethylsilyl (BO-TMSi) and trimethylsilyl (TMSi) derivatives were prepared by reaction with O-benzylhydroxylamine (Aldrich Chemical Co., Milwaukee, Wis. 53210), followed by reaction with N-trimethylsilylimidazole (Pierce Chemical Co., Rockford, Ill. 61105) (*3*, *4*). Methoxime-trimethylsilyl (MO-TMSi) derivatives were prepared in a similar fashion, using O-methylhydroxylamine (*2*). Urinary acid methyl ester (ME) and methyl ester-trimethylsilyl (ME-TMSi) derivatives were prepared by reaction with diazomethane, followed by silylation with BSTFA (*2*). Barbiturate methyl (ME) and methyl-trimethylsilyl (ME-TMSi) derivatives were prepared according to the procedure for urinary acids; the major ME product is the N,N-dimethylbarbiturate, but an appreciable amount of the N,O-isomer(s) and a trace of the O,O-isomer is usually formed as well. The preferred silylating reagent is BSTFA.

Results and Discussion

Urinary Steroid Profile

Derivatization. A new method for obtaining the urinary steroid profile has been developed and is undergoing evaluation for use in studies of adults and the newborn. In this method, reactive ketone groups are converted into BO groups by reaction with O-benzylhydroxylamine, and all hydroxyl groups are converted to TMSi ethers. Steroids containing only hydroxyl groups, or with an unreactive ketone group (11-one), yield TMSi derivatives, while compounds with hydroxyl groups and a reactive ketone group yield BO-TMSi derivatives.

The view that derivative formation is desirable before GC separation of urinary steroids is now generally accepted. It is possible to separate most mono- and difunctional steroids without derivative formation, but for polyfunctional steroids with four, five, or six functional groups, derivative formation is a necessity. Otherwise many components of a urinary sample will be adsorbed and thermally decomposed.

Steroid hydroxyl groups react with silylating reagents at widely different rates. Relatively unhindered groups (3,6,7,15,16,*sec*-17,20,21-ol) react readily, but moderately hindered groups (11*β*,*tert*-17*α* with a 20-one group) react more slowly. The greatest degree of steric hindrance to reaction that we have observed up to the present time is that shown by 17*α*-ol groups in compounds with a 20-ol group. These highly hindered groups are presumably sheltered from reaction by the 20-TMSi ether group. Nevertheless, all hydroxyl groups can be converted into TMSi ethers under appropriate conditions. In earlier systematic studies of this problem (*5*, *6*), we found that the use of N-trimethylsilyl imidazole at 60–80°C resulted in rather slow silylation of the cortols and pregnanetriol; it was later found that they could be completely silylated in a few hours at 150–200°C with this reagent (*7*).

If ketones are converted to BO derivatives they are less volatile than the more frequently used MO derivatives, but an effect of this reaction is to provide a marked separation between TMSi and BO-TMSi derivatives. When this method is applied to adult urinary steroids, four regions of steroids can be recognized in a GC separation with a 1% Dexsil column (Figure 1). Diols and triols are eluted at the start of the steroid profile; major changes occur in this part of the profile during pregnancy, because large amounts of estriol and pregnanediol are present. The second region of the

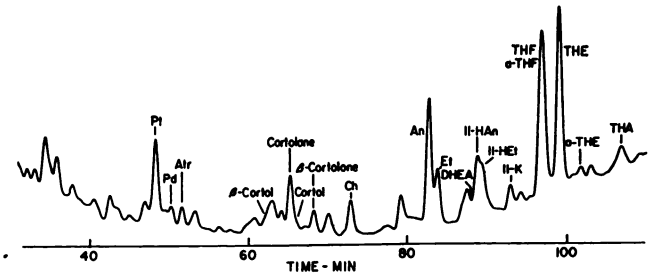


Fig. 1. Metabolic profile of urinary steroids (24-h urine from an adult male) obtained with a 1% Dexsil column by temperature programming (1°C/min)

A procedure was used leading to TMSi and BO-TMSi derivatives, with all hydroxyl groups converted to TMSi ethers. TMSi derivatives: pregnanetriol (*Pt*), pregnanediol (*Pd*), 5-androsten-3β, 16α,17μ-triol (*Atr*), β-cortol, cortolone, cortol, β-cortolone, and cholesterol (*Ch*). BO-TMSi derivatives: androsterone (*An*), etiocholanolone (*Et*), dehydroepiandrosterone (*DHEA*), 11β-hydroxyandrosterone (11-*HAn*), 11β-hydroxyetiocholanolone (11-*HEt*), 11-ketoandrosterone, and 11-ketoetiocholanolone (11-*K*), tetrahydrocortisol (*THF*) allo-tetrahydrocortisol (a-*THF*), tetrahydrocortisone (*THE*), 5α-pregnan-3α,11β,21-triol-20-one (*a-THB*), and 5β-pregnan-3α,21-diol-11,20-dione (*THA*)

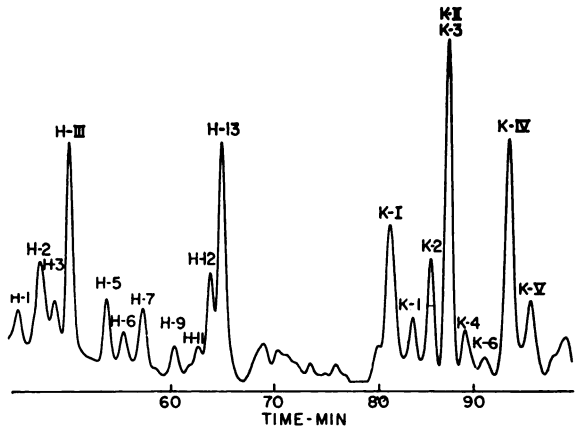


Fig. 2. Metabolic profile of urinary steroids (24 h) from an infant on day 5

The separation of TMSi and BO-TMSi derivatives was carried out by temperature programming at 1°C/min with a 1% SE-30 column. TMSi derivative: *H-III*, 5-androsten-3β,16α,17β-triol. BO-TMSi derivatives: *K-I*, 5-androsten-3β, 16α-diol-17-one; *K-II*, 5-androsten-3β, 17β-diol-16-one; *K-IV*, 5-pregnen-3β,16α-diol-20-one. The *H* series includes other unidentified TMSi derivatives; the *K* series includes other unidentified BO-TMSi derivatives

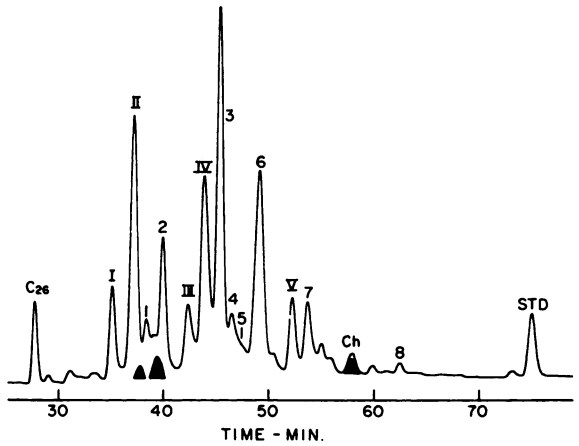


Fig. 3. Metabolic profile of urinary steroids (24 h) from an infant on day 2

This separation of TMSi and MO-TMSi derivatives should be compared with the separation of TMSi and BO-TMSi derivatives in Figure 2. Derivatives with Roman numerals are the same as those in Figure 2; other steroids are unidentified. It is not possible to distinguish hydroxysteroids (with or without an 11-one group) from those containing a reactive ketone group; this difference is evident in Figure 2

profile contains the cortols, cortolones, and cholesterol. The third region contains BO-TMSi derivatives of the 17-keto group of steroids, which includes the 11-substituted 17-ketosteroids in urine. The final region contains BO-TMSi derivatives of pregnanes derived from the adrenocortical steroids and containing a 20-one group. Not all these steroids are fully separated. For example, THF and allo-THF fall in a single peak, and 11-ketoandrosterone and 11-ketoetiocholanolone are not separated.

Variations in profile. Profile alterations that are the result of pathologic conditions or of drug effects may be recognized by changes in the pattern of metabolites. Individual patterns show very little day-to-day variation under ordinary circumstances, but there is interindividual variation. Sex-related differences may be noted; the androsterone/etiocholanolone ratio in Figure 1 is usually reversed for females.

Newborns. This method is valuable in studies of the urinary steroids of the newborn. Figure 2 shows the steroid profile for a newborn on day 5 of life; the division of the profile into two regions is evident. Steroids labeled *H* contain hydroxyl groups (a non-reactive ketone group may or may not be present), while those labeled *K* have a reactive ketone group in addition to hydroxyl groups. Not all of these compounds have been identified, and so their biologic function is unknown. They originate in fetal adrenal tissue; this tissue is lost after birth, but at widely varying rates for different infants, judging from the changes observed in the profiles.

It is also possible to obtain profiles for the newborn by using TMSi derivatives alone, as we did in earlier studies. Figure 3 shows a urinary steroid profile for a 2-day-old infant. TMSi derivatives were separated with an SE-30 column; it is not possible, however, to distinguish the steroid classes. A comparison of Figures 2 and 3 shows the advantage of the BO-TMSi procedure in providing information about structure.

Many variations occur in these steroid profiles at birth, and the rate of change also varies during the first few weeks to months of life. An observation that may prove to be important concerns the estrogens generated by the feto-placental unit; when pooled urine samples are used, it is almost always possible to find peaks for estriol, estrone, and estetrol (1,3,5(10)-estratrien-3,15α,16α,17β-tetrol). When individual samples are studied, it is found that a few infants excrete estrogens, but, for

most, these compounds are negligible contributors to the steroid profile. Exposure of the fetus and the newborn to varying concentrations of estrogens may affect developing tissue.

Urinary Acids

The number of acidic compounds in urine is unknown, but certainly large, and ordinary packed columns with 5000–7000 theoretical-plate efficiency can separate relatively few of these substances. Nevertheless, metabolic profiles of urinary acids are useful for characterizing some pathologic circumstances, and for studying metabolic pathways of drugs and other exogenous materials. Figures 4, 5, and 6 show results obtained through the use of three different procedures, each of which may be appropriate for specific studies.

Figure 4 shows a urinary acid profile obtained with an extract (ether and ethyl acetate) of acidified urine. The extract was treated with diazomethane to form ME, and with a silylating reagent to convert all hydroxyl groups to TMSi ethers. This procedure yields a profile that contains aromatic acids, along with many other acids, and in early studies it was regarded as being useful for detecting abnormal concentrations of aromatic acids (vanilmandelic and 5-hydroxy-indoleacetic acid, for example). While the method is a very simple one, its disadvantages are apparent from the profile. Large amounts of highly water-soluble acids are present in the extract as well as the less soluble aromatic acids, and even urea and ingested caffeine will appear in the profile. Acids like citric acid, for example, are extracted to a varying but incomplete extent, depending upon the conditions of the extraction. It would be more desirable to use a procedure such that the sample would contain all acids, or, alternatively, one that would eliminate most of the highly water-soluble acids.

Figure 5 shows a urinary acid profile containing all acids. The analytical sample was isolated by column separation with DEAE–Sephadex, and ME and ME-TMSi derivatives were prepared as usual. The major components are citric and hippuric acids. A number of highly water-soluble acids that are products of carbohydrate metabolism are present. These include erythronic and threonic acids; the lactones of these acids are also present and were evidently formed after separation of the acid fraction. Other acids include tartaric, isocitric, glucuronic, and saccharic acids. Aromatic acids are also present, but are seen as less prominent peaks because of the higher concentration of polyhydroxyacids. This profile procedure is preferred over that leading to Figure 4, but it has not

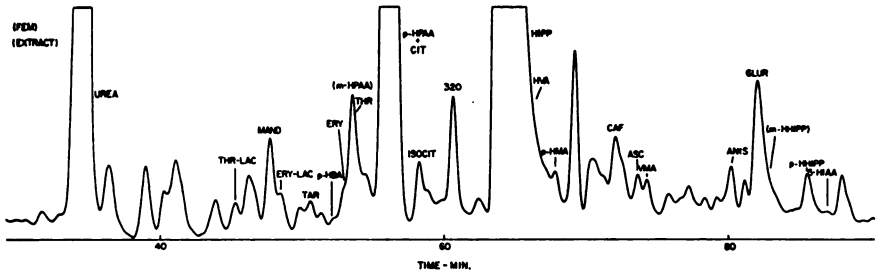


Fig. 4. Metabolic profile of urinary acids (24-h urine from an adult female), as ME and ME-TMSi derivatives

The GC separation was done with a 5% SE-30 column, temperature programmed at 2°C/ min. The analytical sample was obtained by direct extraction with ethyl acetate and ether; neutral substances are present as well. Highly water-soluble acids are incompletely extracted. Identified compounds include threonic acid lactone, *THR-LAC*; mandelic acid, *MAND*, erythronic acid lactone, *ERY-LAC*; tartaric acid, *TAR*; *p*-hydroxybenzoic acid, *p-HBA*; erythronic acid, *ERY*; threonic acid, *THR*; *p*-hydroxyphenylacetic, *p-HPAA*; citric, *CIT*; isocitric, *ISOCIT;* hippuric, *HIPP*, homovanillic, *HVA*, *p*-hydroxymandelic, *p-HMA*; caffeine, *CAF* ascorbic acid, *ASC*; vanilmandelic, *VMA*; glucuronic acid, *GLUR;* anisic acid, *ANIS;* *p*-hydroxyhippuric acid, *p-HHIPP*; 5-hydroxyindoleacetic acid, 5-*HIAA*. Identification of m-hydroxyphenylacetic acid (*m-HPAA*) and *m*-hydroxyhippuric acid (*m-HHIPP*) is tentative. Urea is also present as a TMSi derivative

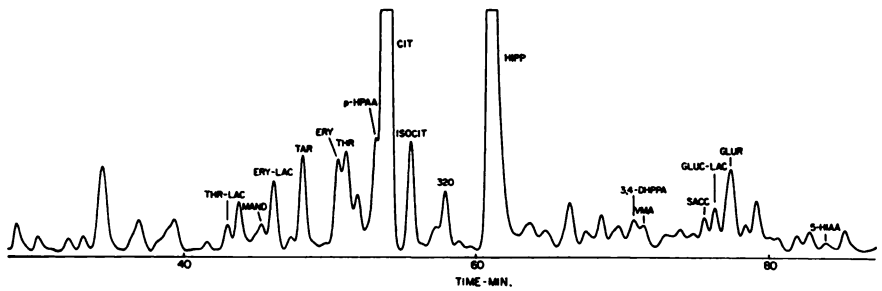


Fig. 5. Metabolic profile of urinary acids (24-h urine from an adult female), same specimen as used for Figure 4

The ME and ME-TMSi derivatives were separated by temperature programming at 2°C/min, with use of a 5% SE-30 column. The analytical sample was obtained by DEAE-Sephadex column isolation procedure, and contains all highly water-soluble acids. The acids are identified as in Figure 4, with the addition of 3,4-dihydroxyphenylpropionic acid, 3,4-*DHPPA*; saccharic acid, *SACC*; gluconic acid lactone, *GLU-LAC*. The lactones are apparently formed after isolation of the acid fraction

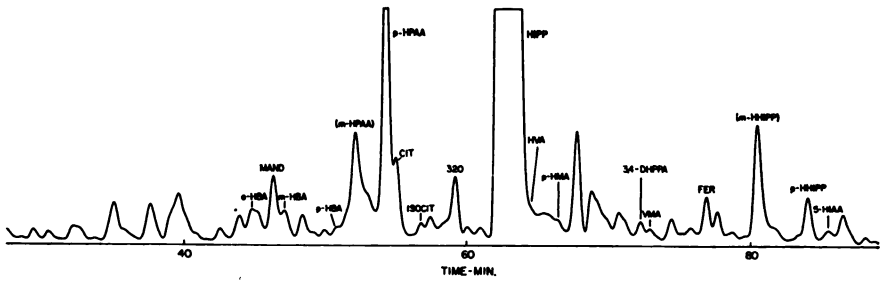


Fig. 6. Metabolic profile of urinary acids (24-h urine from an adult female), same specimen as used for Figures 4 and 5

The ME and ME-TMSi derivatives were separated by temperature programming at 2°C/min, with use of a 5% SE-30 column. The analytical sample was obtained by DEAE-Sephadex column isolation of an acid fraction, followed by an ether–ethylacetate extraction of an acidified aqueous solution of the fraction. This procedure may be regarded as yielding an "aromatic acid" profile; most of the polyhydroxy aliphatic acids are present only in low concentration. In addition to the compounds included in Figures 4 and 5, the acids are: *o*-hydroxybenzoic, *o-HBA*; *m*-hydroxybenzoic, *m-HBA*; *p*-hydroxybenzoic, *p-HBA*; ferulic, *FER*. Compound 320 (mol wt 320) has not been identified

been evaluated to determine its usefulness in studies of disturbances in carbohydrate or amino acid metabolism.

Figure 6 shows a urinary acid profile emphasizing aromatic acids. An acid fraction was isolated as for Figure 5, and an aqueous solution of the fraction was successively extracted with ether and ethyl acetate. The concentration of most of the highly water-soluble acids is greatly decreased when this procedure is followed, and the resulting profile is largely that of aromatic acids. Hippuric and *p*-hydroxyphenylacetic acids are the major components; citric and isocitric acids are present in low concentration. Mandelic, *p*-hydroxymandelic, vanilmandelic, and 5-hydroxyindoleacetic acids are also present, and small peaks may be found for *o*-, *m*-, and *p*-hydroxybenzoic acids and for *p*-hydroxyhippuric acid. The identity of two hydroxyacids, *m*-hydroxyphenylacetic acid and *m*-hydroxyhippuric acid, is less certain (names in parentheses in the Figure). 3,4-Dihydroxyphenylpropionic acid is also present. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is probably of dietary origin.

Abnormal conditions that greatly increase the concentration of specific aromatic acids will lead to the expected changes in the profile. New peaks will be seen when certain types of drugs are ingested. Aspirin, for example, is metabolized to yield multiple products including *o*-hydroxybenzoic, *o*-hydroxyhippuric and gentisic (2,5-dihydroxybenzoic) acids.

Conjugated glucuronides. When these are to be studied, it is best to use the DEAE–Sephadex isolation procedure, and to use a 1% SE-30 column for the separation. Conjugates can be separated without prior hydrolysis; it is, in fact, desirable to study conjugates directly in most instances.

Drugs and Drug Metabolites

Profile procedures may be used in studies of drugs and drug metabolites. The usual alteration in a blood or urine profile resulting from drug administration is that new peaks appear. If the drug(s) and (or) its metabolites are present in relatively high concentration, the changes are usually immediately apparent. If concentrations are low, simply inspecting the profile may not lead to detection of foreign compounds. To detect and estimate drugs and drug metabolites at both relatively high and low concentrations, it is necessary to combine ordinary GC profile methods with the detection capabilities of the mass spectrometer. This is best done with an instrumental system that at present includes a gas chromatograph, mass spectrometer, and computer in a GC–MS–COM system. This instrumental system is more than a collection of instruments: it is in effect a new instrument or, rather, a synergistic instrument system—its capabilities far exceed those of each instrument separately.

Combined instrumentation. Figure 7 shows a profile of urinary metabolites in a rat administered phenobarbital. This was obtained with a GC–MS–COM system based on a Finnigan quadrupole mass spectrometer equipped with a chemical ionization source. The mode of operation was repetitive scan. The chart is a reconstructed gas chromatogram, based on total ion current, resulting from approximately 360 consecutive scans of a temperature-programmed GC separation. All major peaks were identified from their mass spectra. Figure 8 shows the chemical ionization mass spectrum obtained for the first peak in the separation. As part of the analytical procedure, the isolated sample was treated with diazomethane and then with BSTFA, so that barbiturates would be found as N,N-dimethyl derivatives and all hydroxyl groups in metabolites would be converted to TMSi ethers. The spectrum is that of the N,N-dimethyl derivative of phenobarbital. When this chemical procedure is used, some of the drug also yields the N,O-isomer, and Figure 9 shows a chemical ionization mass spectrum for a later peak that is identical with that obtained separately for

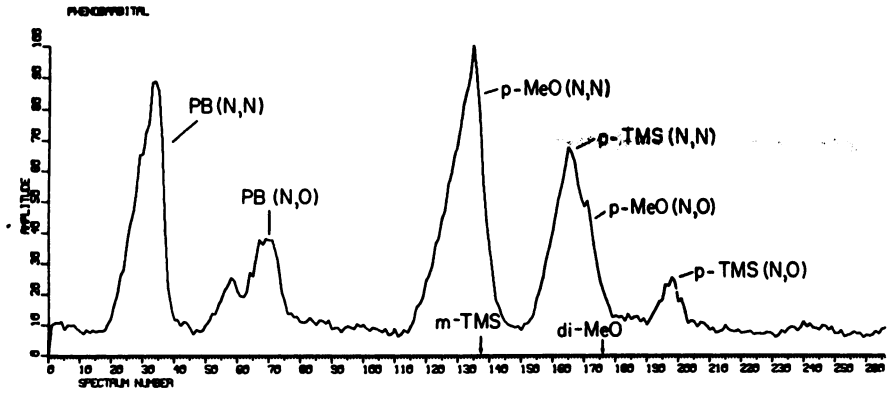


Fig. 7. Metabolic profile of neutral drug metabolites in urine from a rat dosed with phenobarbital

The ME and ME-TMSi derivatives were analyzed with a GC–MS–COM system. The column separation was programmed at 4°C/min with a 1% SE-30 column. The MS unit (Finnigan quadrupole) was operated in repetitive scan mode (about 3 s for 50–500 amu scan). A chemical ionization source was used with methane as the carrier gas. The chart, drawn by the COM unit, is a reconstructed gas chromatogram based upon total ion yield. The compounds are: phenobarbital, as the N,N-dimethyl derivative, *PB(N,N)*; phenobarbital, as the N,O-dimethyl derivative, *PB(N,O)*; *p*-hydroxyphenobarbital, as the N,N ME derivative, *p-MeO(N,N)*; p-hydroxyphenobarbital, as the N,O ME derivatives, *p-MeO(N,O)*; *p*-hydroxyphenobarbital, as the N,N ME-TMSi derivative, *p-TMS(N,N)*; *p*-hydroxyphenobarbital, as the N,O ME-TMSi derivatives, *p-TMS(N,O)*; *m*-hydroxyphenobarbital, as the N,N ME-TMSi derivative, *m-TMS*; a dihydroxyphenobarbital, as the N,N ME derivative containing four methyl groups, *di-MeO*

the isomer. The major metabolite is *p*-hydroxyphenobarbital, and the conditions of derivative formation were chosen so that both O-ME and O-TMSi derivatives would be present. Figure 10 shows the mass spectrum for another peak, identified as the ME derivative of *p*-hydroxyphenobarbital, and Figure 11 shows the corresponding spectrum for the ME-TMSi derivative. The peaks are identified in Figure 7.

When reference compounds are available, drugs and their metabolites can be identified unequivocally by a combination of data from GC and MS, obtained in this way. The detection of compounds present in low concentration, which may not have

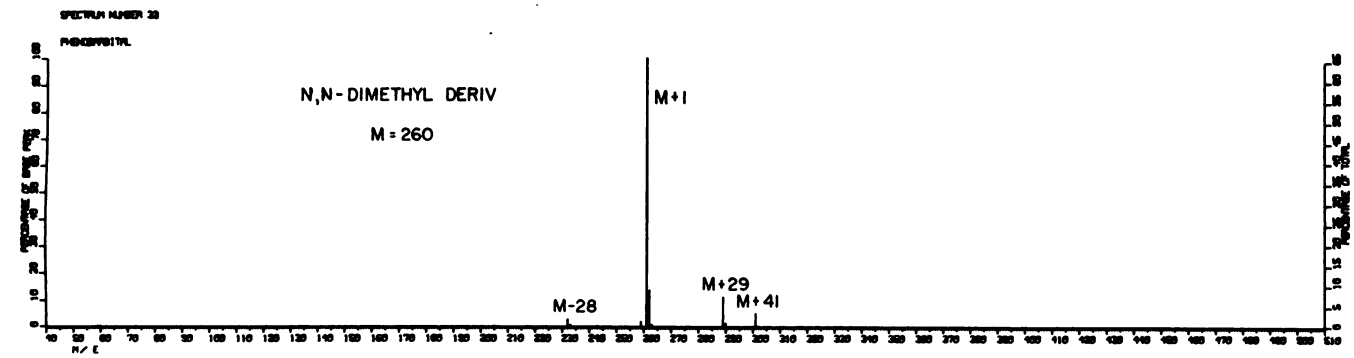


Fig. 8. Chemical ionization mass spectrum of the N,N ME derivative of phenobarbital (M = 260), taken as spectrum No. 33 from the separation in Figure 7

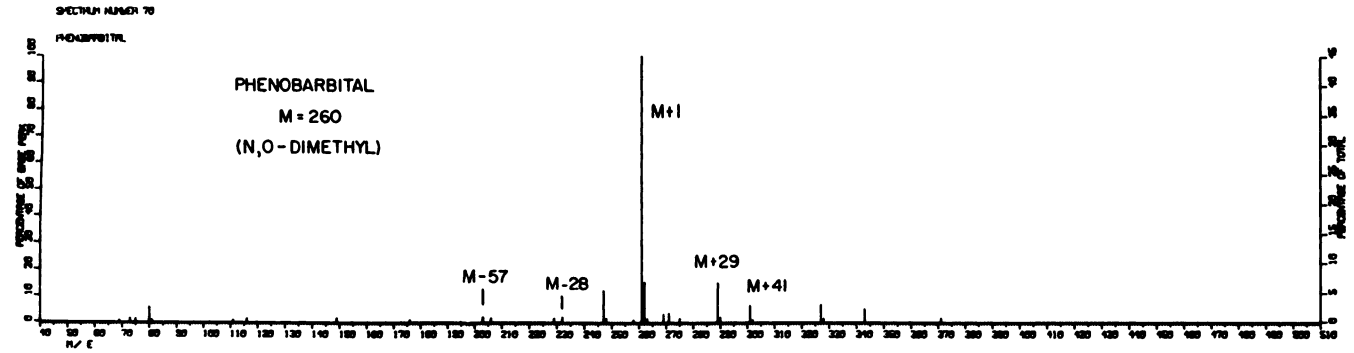


Fig. 9. Chemical ionization mass spectrum of the N,O ME derivatives of phenobarbital (M = 260), taken as spectrum No. 70 from the separation in Figure 7

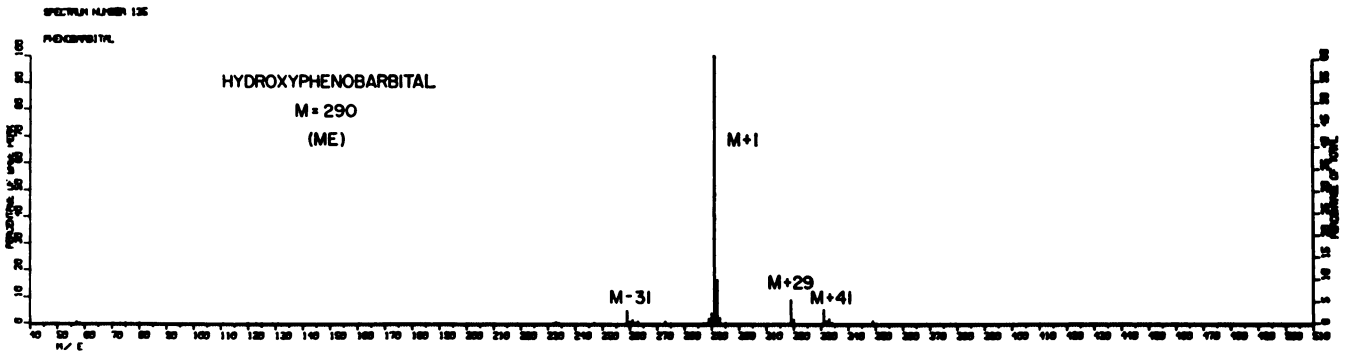


Fig. 10. Chemical ionization mass spectrum of the N,N ME derivative of *p*-hydroxyphenobarbital (M = 290), taken as spectrum No. 135 from the separation in Figure 7

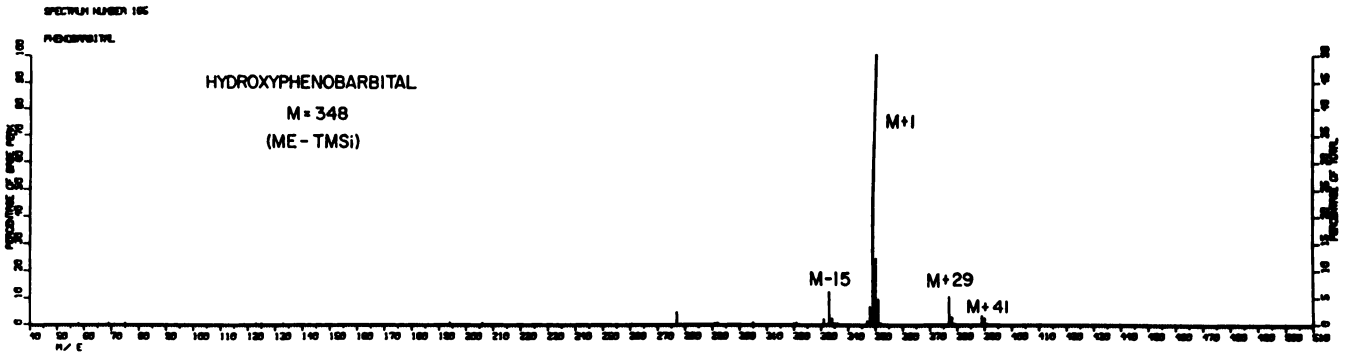


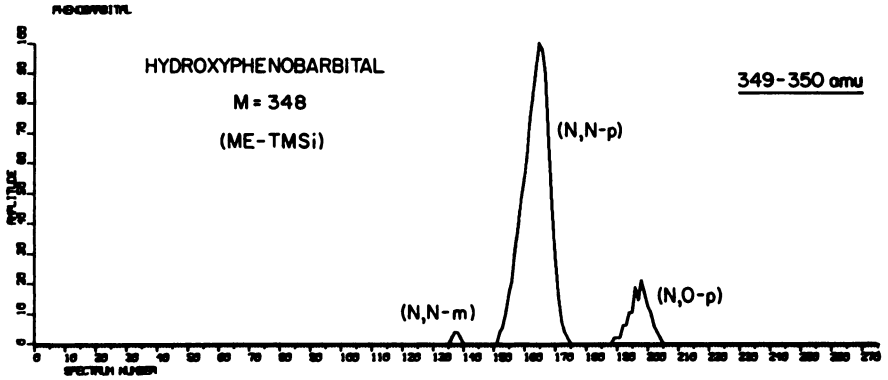
Fig. 11. Chemical ionization mass spectrum of the N,N ME-TMSi derivative of p-hydroxyphenobarbital (M = 348), taken as spectrum No. 165 from the separation in Figure 7

been observed previously, and which are not evident in the profile separation, presents a different problem. By programming techniques, it is possible to search each spectrum for characteristic ions. These may be known from previous work, or they may be predicted from a knowledge of ion-molecule reactions occurring in the mass spectrometer. Figure 12 shows a record resulting from the search of all spectra for ions at 349–350 amu, corresponding to MH or (M + 1) = 349 for the ME-TMSi derivative of a monohydroxyphenobarbital. Three peaks are evident. The N,N- and N,O-isomers were evident in the profile separation (Figure 7), the small peak, which is not visible, is believed to be the N,N-derivative of *m*-hydroxyphenobarbital. When a search was made for the ME derivative of dihydroxyphenobarbital, the result in Figure 13 was obtained. The position of these trace components is noted in Figure 7 with arrows.

These methods may be extended to the detection and estimation of trace components of all kinds, if the ions used for identification are not produced by other compounds eluting at the same time. Profile separations similar to that in Figure 12, for example, can be used to detect compounds not evident in the ordinary mass detection profile. A GC–MS–COM system provides a capability for selective detection at high sensitivity, for use in profile studies.

Fig. 12. Reconstructed gas chromatogram, based upon the separation in Figure 7, and showing the occurrence of compounds yielding ions at 349–350 amu

In addition to the expected N,N and N,O ME-TMSi derivatives of *p*-hydroxyphenobarbital, a small amount of another derivative was found. This has been tentatively identified as the isomeric N,N ME-TMSi derivative of *m*-hydroxyphenobarbital



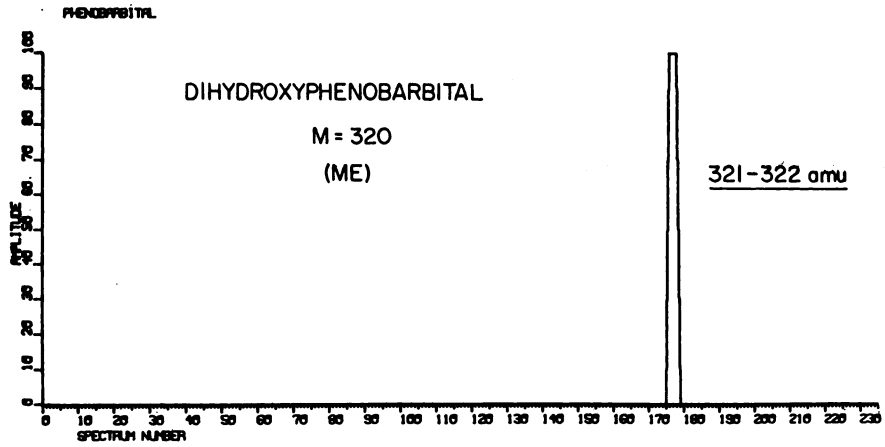


Fig. 13. Reconstructed gas chromatogram, based on the separation in Figure 7, and showing the occurrence of a compound yielding ions at 321–322 amu. This has been tentatively identified as the N,N ME derivative of 3,4-dihydroxyphenobarbital

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