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AUTOMATED QUALITATIVE AND QUANTITATIVE METABOLIC PROFILING ANALYSIS OF URINARY STEROIDS BY A GAS CHROMATOGRAPHY–MASS SPECTROMETRY–DATA SYSTEM

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SUMMARY

A computer system (MSSMET), using methylene unit retention indices for an off-line reverse library search analysis of selected ion chromatograms from gas chromatography–mass spectrometry data, has been applied to the qualitative and quantitative determination of urinary steroids. Several published methods for the isolation and derivatization of urinary steroids were evaluated for reproducibility using fused silica capillary column gas chromatography. Using a procedure that gave the greatest reproducibility, MSSMET analyses of urinary steroids were evaluated with packed (3-m 3% OV-101) and capillary (50-m OV-101 WCOT fused silica) columns. Most urinary steroids could be accurately quantitated using the packed column. However, urinary steroids with similar mass spectra and retention behavior on a packed column (*i.e.*, androsterone and etiocholanolone, or 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one and 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one) were completely separated using the capillary column and could be reproducibly quantitated with a 2-sec scan cycle time (10–15 data points across a peak) but not with a longer scan cycle time. Overloading was the major problem encountered with the fused silica capillary column.

INTRODUCTION

The concept that individuals have distinct metabolic patterns reflected by the constituents of their biological fluids (*i.e.*, urine, blood, amniotic fluid, cerebrospinal fluid, sweat, etc.) originated with the work of Williams[1]. By utilizing the technique of paper chromatography Williams showed convincingly that the patterns for a variety of compounds found in urine varied greatly from one individual to another but were relatively constant for any given individual. Williams' concept was not employed by others until the late 1960's when liquid and gas chromatographic (GC) techniques had become sufficiently refined for studies of this type. The phrase most often used to describe multicomponent analyses of biological fluids, "metabolic profiling", was

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defined by Horning and Horning[2,3] as "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites". The Hornings also suggested that "profiles may prove to be useful for characterizing both normal and pathologic states, for studies of drug metabolism, and for human development studies".

Although metabolic profiling analysis of biological mixtures is a relatively new technology, the analytical techniques are well known. They include paper chromatography, thin-layer chromatography, column chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Of these techniques, the most versatile and useful is GC–MS with computer-assisted analysis of the data (DS). Indeed, most current research in the area of metabolic profiling analysis uses the technique of computer reconstructed ion chromatograms from arrays of complete mass spectra[4].

Metabolic profiling of human urinary steroids has attracted much more attention in the past 10 years, mostly from investigators in Europe, and the volume of literature on this subject is large. Among the more obvious applications of this technique in the steroid field are studies of individuals with defects in steroid metabolism (*i.e.*, Cushing's syndrome, adrenal carcinoma, pituitary tumors, 21-hydroxylase deficiency, 17α-hydroxylase deficiency, etc.) and many examples of steroid profiles have appeared in the literature[5–11]. Steroid metabolic profiles have also been studied in pregnancy[12,13], in newborns and infants[14,15], and in conditions of emotional stress and physical exertion[10]. Steroid metabolic profiles of neutral and acidic steroids have also been obtained for blood, amniotic fluid, bile and feces[16,17].

Metabolic profiling analysis of urinary steroids originated with the work of Gardiner and Horning[18], who demonstrated that most of the major urinary steroids could be separated in a single GC run as the methoxime-trimethylsilyl ether derivatives. The major advances since this time have involved improvements in the methods for isolation of steroids from urine, serum, or tissue[19–24], the use of wall-coated open-tubular (WCOT) glass capillary columns[5–7,25,26], and the use of computer-assisted GC–MS analysis of steroid profiles[27–31].

Automated analysis of steroid profiles by GC–MS–DS involves three stages of analysis: (1) preparation of derivatized samples; (2) GC–MS analysis and (3) computer analysis of the data. Extraction of steroids from urine, serum or tissues can be either general, or specific groups of steroids can be selectively isolated and purified. Introduction of the Amberlite XAD-2 extraction procedure by Bradlow[22] greatly simplified the extraction of polar steroids and steroid conjugates. Various procedures for general extraction of steroids from tissues and body fluids using solids and non-polar solvents have appeared in the literature[19,21,22,24]. Selective isolation procedures for estrogens, 3-keto steroids, synthetic steroids possessing an ethynyl group, free steroids, steroid glucuronides, steroid sulfates and steroid disulfates using column liquid chromatography and solvent extraction procedures have appeared in the literature[23,24,32–35]. Sjovall, Axelson and co-workers have done much of the pioneering research on these purification procedures[23,24,28,33] and on automated analysis using a forward library search technique for the qualitative identification of individual components of mixtures of steroids[23,29,36].

This report concerns the development in our laboratory of a suitable procedure for the analysis of steroids from urine with a previously described GC–MS computer system for automated qualitative and quantitative analysis by a reverse library search technique[37–39].

EXPERIMENTAL

Materials

All solvents were redistilled in glass. Enzymes used were β-glucuronidase from *Helix pomatia* [activity: 62880 Fishman units (F.U.) per vial, with one F.U. defined as that activity which will hydrolyze 1.0 mg of phenolphthalein glucuronide per hour at pH 5.0, 37°C; Calbiochem, La Jolla, CA, U.S.A.]. Amberlite XAD-7 (polystyrene non-ionic adsorbent; Mallinckrodt, Paris, KY, U.S.A.) was washed with ten volumes of methanol, acetone, methanol–distilled water (1:1), ethanol and finally distilled water. O-Methoxyamine hydrochloride and Sylon BTZ [N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)–trimethylchlorosilane (TMCS)-trimethylsilylimidazole, 3:2:3] were purchased from Supelco (Bellefonte, PA, U.S.A.). Reference steroids were purchased from Steraloids (Wilton, NH, U.S.A.). Lipidex-5000 was purchased from Packard Instruments (Downers Grove, IL, U.S.A.).

Preparation of standards

Each steroid standard (2–5 mg) was weighed into a 2-dram screw-top vial with a PTFE cap liner. Methanol was then added to make a 1.0 μg/μl solution. Samples which contained undissolved steroids were diluted with an equal volume of dimethylsulfoxide (DMSO), making a 0.5 μg/μl solution. An aliquot (250 μl) of each stock solution (500 μl for those with DMSO added, making a total of 250 μg per sample) was placed in a 10-ml test-tube with a screw-top cap (PTFE-lined) and the solvent was evaporated with a stream of nitrogen at ambient temperature. Methoxime-trimethylsilyl (MO-TMS) derivatives were prepared as follows: 50 μl of a 100 μg/μl solution of O-methoxyamine hydrochloride in dry, redistilled pyridine were added and this mixture was heated at 60°C for 1 h. Following removal of excess reagent under a stream of nitrogen, 250 μl of Sylon BTZ were added and the samples were heated at 80°C for 18 h. Samples were placed in glass capillaries which had previously been sealed at one end and then sealed with a flame. Retention indices (*i.e.*, methylene units) for each MO-TMS derivative were determined on a 25-m SP-2100 wall-coated open tubular capillary (WCOT) column (0.2 mm I.D.) and on a 3-m, 3% OV-101 [Supelcoport 80–100 mesh, 2 mm I.D.] packed column by co-injection of straight-chain saturated hydrocarbons. Each sample was also injected separately to insure that the derivatization procedure was quantitative (*i.e.*, only one peak was observed).

Isolation of steroids

Amberlite XAD-7 was packed in 1.0-cm I.D. glass columns to a height of 4 cm. Each column had a 100-ml reservoir. Columns were washed with 30 ml of distilled water before being used. Urine samples (20 ml each) were pipetted onto the columns. The loaded columns were washed with 2 × 5 ml of distilled water. Steroids were eluted with 3 × 5 ml of absolute ethanol, at a constant flow-rate (0.5–1.0 ml/min). Ethanol was evaporated from the eluate by aspiration using a rotary evaporator in a water-bath at 37°C. A sodium acetate buffer (0.5 *M*, pH 4.55) was added (3 ml, plus 1 ml to wash) and the samples were transferred to small erlenmeyer flasks. Cholesteryl butyrate was added (1 μg/μl; 20 μl per sample) as an internal standard. β-Glucuronidase, derived from mollusk (Calbiochem) by adding 3 ml of distilled water to each vial, was added (300 μl) to each sample. After incubation for 48 h in a 37°C water-

bath, sodium chloride (1.5 g) was added. Liberated steroids and steroid sulfates were extracted by shaking with 25 ml of ethyl acetate for 60 min. The ethyl acetate layer was removed by pipet, and the aqueous phase was re-extracted two more times with 5-ml aliquots of ethyl acetate. The pH of the aqueous phase was adjusted to 1.0 with concentrated HCl and the solution was extracted again with ethyl acetete. The combined organic phases were heated at 45°C for 18 h and then combined with the first extraction phase. The recombined ethyl acetate phase was washed with 2 × 5 ml of 8% aqueous sodium bicarbonate then transferred to a small round-bottom flask (washing the aqueous phase three times with 2-ml aliquots of ethyl acetate). Steroids lost in the aqueous phase were recovered by XAD-7 extraction and elution with ethanol, as described above.

The combined ethyl acetate and ethanol phases were evaporated by aspiration using a rotary evaporator and a water-bath at 37°C. The samples were resuspended in 3 ml of ethanol and transferred to small screw-top test-tubes (washing with 2 × 1 ml ethanol). Samples were then dried under a stream of nitrogen at 60°C, and 50 μl of a solution of O-methoxyamine hydrochloride (100 μg/μl) in dry pyridine, plus 100 μl of dry pyridine, were added to the dry residue. This solution was heated for 60 min at 60°C, excess pyridine was removed under nitrogen at 60°C, 100 μl of Sylon BTZ were added, and the sample was heated for 18 h at 80°C.

Excess silylation reagents and compounds with polarities similar to those of steroids were separated from the steroid fraction on a Lipidex-5000 column (70 × 4 mm) containing hexane–hexamethyldisilazane–pyridine–2,2-methoxypropane (97:1:2:10, v/v). The sample was transferred to the top of the column by adding 400 μl of the solvent, which was also passed through the column. For rapid filtration nitrogen pressure was applied, resulting in a flow of 3 ml/min. Solvent (3.5 ml) was passed through the column to recover the derivatized compounds. Solvents were then evaporated under nitrogen at 60°C and the sample was redissolved in 1 ml of hexane containing 2% BSTFA.

Gas chromatography

Gas chromatography was performed on a Hewlett-Packard 5840A equipped with a split and splitless capillary column injection port and a flame ionization detector. Aliquots (1 μl) of the trimethylsilylation reaction mixture were chromatographed on a SP-2100 wall-coated open tubular (WCOT) fused silica capillary column (25 m × 0.2 mm I.D.). Conditions of analysis were as follows: injection port temperature, 280°C; initial temperature, 180°C; temperature programming at 2°C/min to a final temperature of 280°C; 50 min isothermal period at the end of the run; detection signal attenuation, 1; hydrogen flow, 30 ml/min; and air flow, 200 ml/min; split ratio, 20:1.

Mass spectrometry

Mass spectral data were obtained on an LKB-2091 gas chromatograph–mass spectrometer with a dual Digital Equipment PDP-8e based foreground–background data system[12]. The gas chromatograph contained a coiled glass column (3 m × 2 mm I.D.) packed with 3% OV-101 on Supelcoport (80–100 mesh), a 50-m OV-101 WCOT fused silica capillary column (0.3 mm I.D.) or a 25 m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.). Conditions of operation were as follows: GC oven temperature programming from 180 to 280°C at 2°C/min; ion source temperature,

280°C; GC injection port, 280°C; electron multiplier voltage, 1500 V; scans at 4-sec intervals at scan speed 3 (m/z 50–730) or 2-sec intervals at scan speed 2 (m/z 50–600); accelerating voltage, 3.5 kV; trap current, 50 μA; filament current, approximately 4 A; and ionizing voltage, 70 eV. Calibration of nominal mass was against reference ions of perfluorokerosene and instrumental performance was evaluated each day by inspection of reconstructed mass chromatograms. The GC column was pre-treated by two injections of BSTFA–TMCS silylation mixture at 280°C. An aliquot (0.5 μl) of a mixture of eight straight-chain saturated hydrocarbons (20, 22, 24, 26, 28, 30, 32 and 34 carbon atoms) in hexane was co-injected with each sample. After samples were analyzed under the above conditions, each run was validated by brief manual inspection of a few selected ion chromatograms and the data were then transferred to a PDP 11/40 computer (Digital Equipment) for subsequent analysis and storage on magnetic tape. The PDP 11/40 system consisted of a 16-bit, 124 K-word memory minicomputer with two 1.2 million word removable disks, one CDC-9766 300 Mega byte disk, a seven-track magnetic tape drive, DEC writer, Tektronix 4010 scope display unit, and a Tektronix 4610 hard copy unit.

RESULTS AND DISCUSSION

Various methods for the isolation and derivatization of urinary steroids were investigated for reproducibility. The aim of these investigations was to establish the simplest "general" extraction procedure that would give a high degree of reproducibility. The first procedure we investigated used an XAD-2 extraction, followed by enzymatic hydrolysis and finally extraction of the steroids a second time with XAD-2. Trimethylsilylimidazole was used to derivatize the extracted steroids. We were unable to obtain a reproducible capillary column GC trace with or without a Lipidex-5000 "clean-up" step (see Experimental) using this procedure. Leunissen and Thijssen[19] reported overall recoveries of 90% or better of the radioactivity when human subjects were administered labeled androstenedione, estrone, 3β-hydroxy-5-androstene-17-one (DHEA) and DHEA sulfate. They also reported a high degree of precision, which we have confirmed (Fig. 1). Our investigations have also confirmed that the alkali wash and purification of the silylation mixture are important steps for obtaining reproducible GC chromatograms. The precision of this method was about the same with or without the solvolysis step. However, although we only found very small amounts of liberated steroids in the solvolysis phase in two different urine samples, this step was included in the procedure to minimize the effect of variations in enzyme activity from one batch to another, and because the sulfatases isolated from *Helix pomatia* reportedly are not able to hydrolyze 3α-OH sulfate conjugates in 5α-steroids and 17- and 20-hydroxyl sulfate conjugates[19].

Quantitative metabolic profiling of volatile derivatives of steroids isolated from tissues or body fluids by GC–MS analysis using the repetitive scanning technique can of course be accomplished using packed or capillary columns. Capillary columns offer two distinct advantages over packed columns, increased resolution and decreased sample loss due to adsorption during chromatography. The major disadvantage of capillary columns is the necessity for shorter repetitive scan cycle times to establish a sufficient number of data points in reconstructed mass chromatograms. Quadrupole mass filters offer sufficiently short repetitive scan cycle times and have

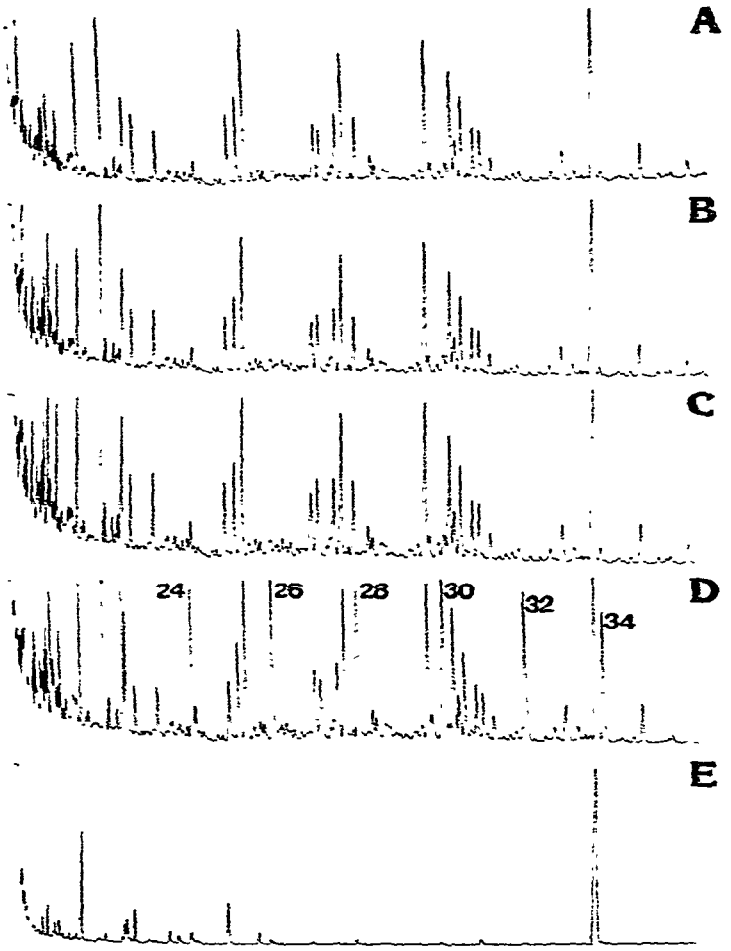

Fig. 1. Urinary steroid metabolic profile of a post-puberal pre-menopausal female, obtained by capillary column GC with flame ionization detection. The top three traces (A, B and C) are for three separate preparations of the same urine sample and demonstrate the overall excellent precision of the method. The lower recordings are of sample plus a mixture of straight-chain hydrocarbons (D), and of a sample blank, using distilled water (E). Conditions of analysis are given in the text.

been used in the analysis of steroid profiles by capillary column GC–MS[30,31]. However, magnetic sector instruments have the disadvantage of slower repetitive scan cycle times. One way to circumvent partially this problem is to shorten the mass range over which the magnet is scanned. For example, Axelson and Sjovall[33] reported using a 4-sec scan cycle time over m/z 250–480 in the analysis of steroids isolated from plasma.

Optimum qualitative and quantitative conditions for automated GC–MS–DS analysis of urinary steroids were established with a 3-m, 3% OV-101 packed column. Although it was known that many of the steroids do not separate satisfactorily by packed column chromatography, selected ion chromatograms were examined for their potential in quantitating poorly-resolved GC peaks. Fig. 2 is a GC–MS total ion

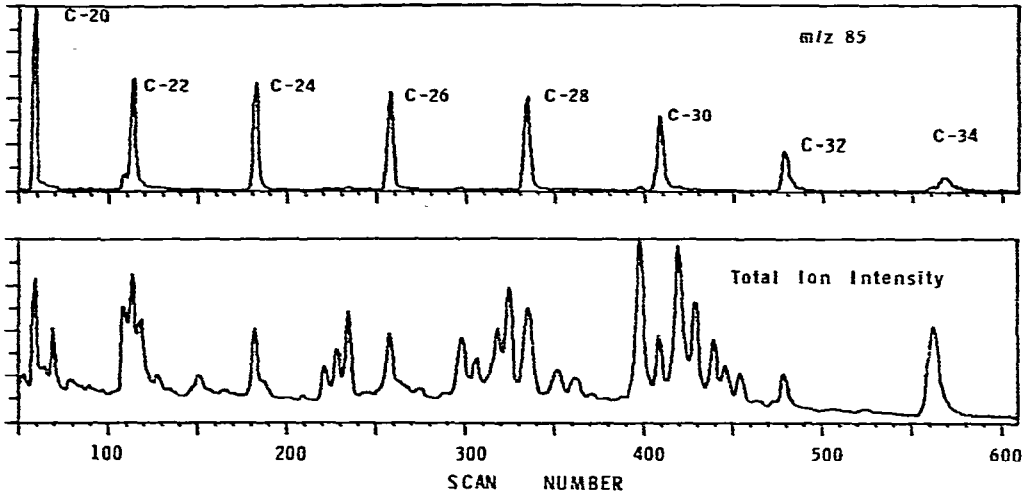

Fig. 2. GC–MS analysis of urinary steroids using a 3-m, 3% OV-101 packed column. Shown are the total ion intensity and the ion chromatogram for m/z 85 which shows where the co-injected hydrocarbons elute. Conditions of analysis are given in the text.

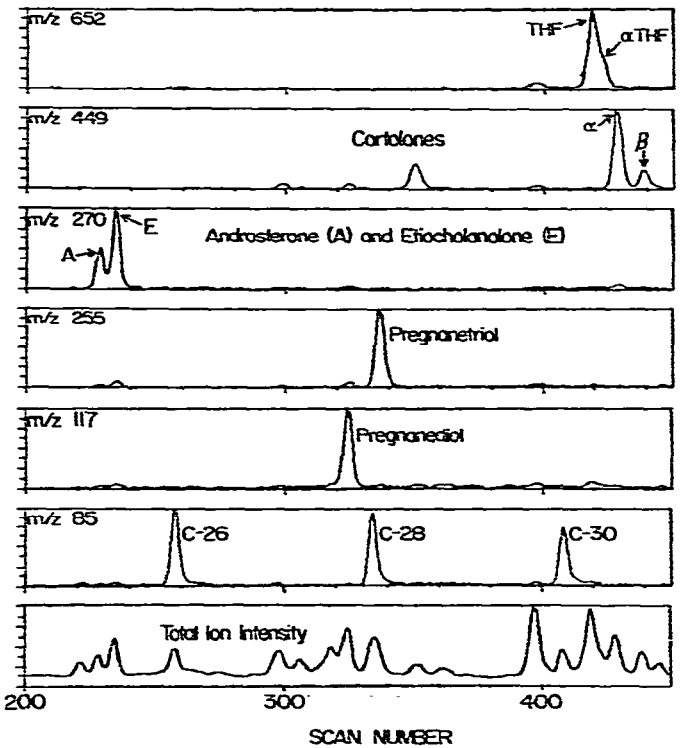

Fig. 3. GC–MS analysis of urinary steroids using a 3-m, 3% OV-101 packed column. Shown are the total ion intensity and an ion chromatogram of a characteristic ion for each of several steroids found in human urine. Conditions of analysis are given in the text.

intensity trace of a sample of urinary steroids separated on the 3-m OV-101 packed column. Conditions of analysis are given in the Experimental. The selected ion chromatogram at *m/z* 85 shows where the co-injected hydrocarbons eluted. Fig. 3 demonstrates that many urinary steroids can be easily quantitated by selected ion chromatography when packed columns are used, while poorly-resolved steroids, which have nearly identical mass spectra, cannot be adequately analyzed by this approach. These results were anticipated from previous work by other investigators.

An alternative approach, using capillary columns for the automated GC–MS-DS analysis of urinary steroids, was then investigated. To quantitate accurately the sharper capillary column peaks the scan cycle time was shortened. Using a 25-m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.) the ability of 1-, 2-, and 3-sec repetitive scan cycle times to reproducibly quantitate the much sharper capillary column peaks using reconstructed ion chromatograms was investigated. Both 1- and 2-sec repetitive scan cycle times gave the desired reproducibility. A 3-sec cycle time was inadequate except when the amount of sample injected was sufficient to overload the column. Fig. 4 shows the total ion intensity obtained using a 2-sec scan cycle time (*m/z* 50–600). The top of Fig. 5 shows the region between C_{24} and C_{30}. Characteristic selected ion chromatograms of the co-injected hydrocarbons, androsterone, etiocholanolone, pregnanediol, pregnanetriol and 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione (THE) are displayed. It is important to understand that these steroids are identified not only by their characteristic ion currents, but also by their characteristic retention indices.

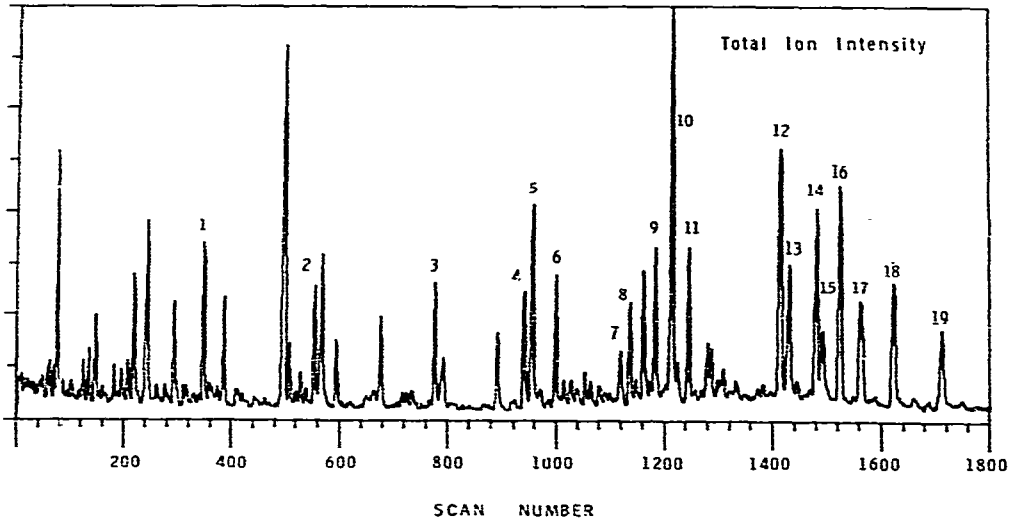

Fig. 4. GC–MS analysis of urinary steroids using a 50-m (0.3 mm I.D.) OV-101 WCOT fused silica capillary column. Shown is the total ion intensity. Three μl of the sample were injected along with 1 μl of a mixture of straight-chain saturated hydrocarbons (0.5 μg/μl). A 5:1 splitting ratio was used. Identity of peaks: 1 = C_{20} hydrocarbon; 2 = C_{22} hydrocarbon; 3 = C_{24} hydrocarbon; 4 = androsterone; 5 = etiocholanolone; 6 = C_{26} hydrocarbon; 7 = 11β-hydroxyandrosterone; 8 = 11β-hydroxyetiocholanolone; 9 = 16α-hydroxydehydroepiandrosterone; 10 = pregnanediol and C_{28} hydrocarbon; 11 = pregnanetriol; 12 = THE; 13 = C_{30} hydrocarbon; 14 = 3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one (THF); 15 = 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one (α-THF); 16 = α-cortolone; 17 = β-cortolone and β-cortol; 18 = α-cortol; 19 = C_{32} hydrocarbon.

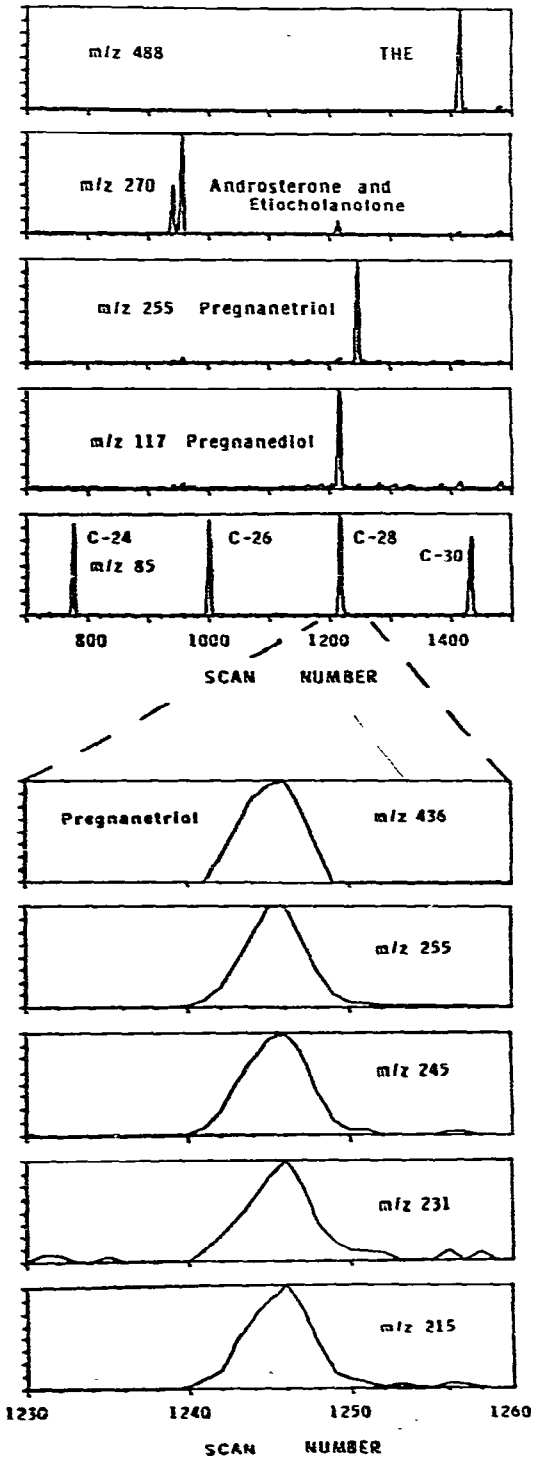

Fig. 5. GC–MS analysis of urinary steroids using a 50-m OV-101 WCOT fused silica capillary column. Shown are characteristic ion chromatograms for the co-injected hydrocarbons, androsterone, etiocholanolone, pregnanediol, pregnanetriol and THE. The bottom graph shows the set of ions for pregnanetriol that the MSSMET program uses to identify and quantitate this compound. Conditions of analysis are given in the text. A *K*-factor (refs. 37 and 39) was determined for pregnanetriol and the amount injected was calculated to be 18 ng.

During the past 5–6 years, a system has been developed at the MSU/NIH Mass Spectrometry Facility for automated simultaneous qualitative and quantitative analysis of complex organic mixtures by GC–MS–computer systems[37,38]. This technique uses methylene unit retention indices for the time dimension and an off-line reverse library search of the data obtained from GC–MS runs for qualitative and quantitative analysis of complex biological mixtures. The system is abbreviated as MSSMET (mass spectral metabolite program).

The bottom of Fig. 5 shows the set of ions for pregnanetriol that the MSSMET program used to identify and quantitate this compound. For each compound searched for by the MSSMET program, one ion that is both characteristic and intense is used for quantitative purposes and the presence and relative intensities of a few other ions produced during fragmentation are used to confirm the identity of the compound in question. Retention index as well as good ion statistics on the designate and confirming ions are crucial in the identification of each compound. In the exampie shown, *m/z* 255 is used to quantitate pregnanetriol and the other ions are used in the calculation of a confidence coefficient indicating the presence of this compound.

Reproducibility of the computer-assisted automated metabolic profiling analysis of urinary steroids using capillary column GC–MS is summarized in Table I. Values are the integrated peak areas determined by the MSSMET program of a characteristic ion for each steroid expressed as a percent of the sum of the areas for these five urinary steroids. Data are from four separate GC–MS analyses of the same sample using a 25-m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.). Data are given for the earlier eluting steroids since their GC peaks are much sharper than those occurring later in the GC run (the cortols and the cortolones for example; see Fig. 5) and thus they are the most difficult to quantitate accurately by the repititive scanning technique. The overall precision was 4.8% for these four separate GC–

TABLE I

PRECISION: CAPILLARY COLUMN GC–MS PROFILING OF URINARY STEROIDS

Values are the integrated peak areas determined by MSSMET of a characteristic ion for each steroid expressed as a percent of the sum of the areas for these five urinary steroids. Data are from four separate GC–MS analyses of the same sample using a 25-m SP-2100 WCOT fused silica capillary column (0.2 mm I.D.).

Steroid	*Run number*				*Mean ± S.D.**
	1	*2*	*3*	*4*	
Androsterone	9.4	8.6	10	10	9.50 ± 0.7
Etiocholanolone	12	12	13	13	12.5 ± 0.6
Pregnanediol	59	59	57	56	57.8 ± 1.5
Pregnanetriol**	14	16	15	15	15.0 ± 0.8
THE	5.4	5.8	5.8	5.9	5.73 ± 0.22
Etio./Andro.***	1.2	1.3	1.3	1.3	1.28 ± 0.05

* The average precision was ±4.8% (calculated by expressing each S.D. as a % of the mean for the five steroids shown).

** A *K*-factor (refs. 37 and 39) was determined for pregnanetriol and the amount injected corresponds to 18 ng.

*** Ratio of etiocholanolone to androsterone.

MS analyses (calculated by expressing the standard deviation as a percent of the mean and averaging these values for the five steroids shown). Also shown in Table I is the ratio of etiocholanolone to androsterone as determined by MSSMET. Using a *K*-factor (calculated by knowing the ratio of the intensities of ion currents for the internal standard, cholesteryl buryrate, and pregnanetriol for equal amounts of each compound; refs. 37 and 39), the amount of pregnanetriol was calculated to be 18 ng injected (150 ng/ml urine). These results demonstrate that a 2-sec scan cycle time is sufficient to quantitate urinary steroids by capillary column GC–MS using reconstructed ion chromatograms from repetitive scanning data.

The investigations just described have led to the development of an automated GC–MS–DS procedure for reproducible quantitative analysis of complex mixtures of steroids. Important features of the system are the use of capillary column chromatography, a non-mass discriminating mass analyzer and a fully automated reverse library search procedure using methylene unit retention indices and reconstructed mass chromatograms.

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