Translation Series No. 4128

Investigation of the steroids in blood with the combination glass capillary column gas chromatography - mass spectrometry

by H. Ludwig, J. Reiner, and
G. Spiteller

Original title: Untersuchung der Steroide im Blut mit der Kombination Glaskapillar-gaschromatographie-Massenspektrometrie .

From: Chem. Ber. 110: 217-227, 1977

Translated by the Translation Bureau (MMA)
Multilingual Services Division
Department of the Secretary of State of Canada

Department of Fisheries and the Environment
Fisheries and Marine Service
Halifax Laboratory
Halifax, N.S.

1977

16 pages typescript

Form 4128

DEPARTMENT OF THE SECRETARY OF STATE
TRANSLATION BUREAU

MULTILINGUAL SERVICES DIVISION

CANADA

SECRÉTARIAT D'ÉTAT
BUREAU DES TRADUCTIONS

DIVISION DES SERVICES MULTILINGUES

TRANSLATED FROM – TRADUCTION DE	INTO – EN
German	English

AUTHOR – AUTEUR
LUDWIG, Helga, REINER, J. and SPITELLER, G.

TITLE IN ENGLISH – TITRE ANGLAIS
Investigation of the steroids in blood with the combination glass capillary column gas chromatography - mass spectrometry.

TITLE IN FOREIGN LANGUAGE (TRANSLITERATE FOREIGN CHARACTERS)
TITRE EN LANGUE ÉTRANGÈRE (TRANSCRIRE EN CARACTÈRES ROMAINS)

Untersuchng der Steroide im Blut mit der Kombination Glaskapillargaschromatographie-Massenspecktrometrie

REFERENCE IN FOREIGN LANGUAGE (NAME OF BOOK OR PUBLICATION) IN FULL. TRANSLITERATE FOREIGN CHARACTERS.
RÉFÉRENCE EN LANGUE ÉTRANGÈRE (NOM DU LIVRE OU PUBLICATION), AU COMPLET, TRANSCRIRE EN CARACTÈRES ROMAINS.

Chem. Ber.

REFERENCE IN ENGLISH – RÉFÉRENCE EN ANGLAIS

Chemical Reports

PUBLISHER – ÉDITEUR	DATE OF PUBLICATION / DATE DE PUBLICATION			PAGE NUMBERS IN ORIGINAL / NUMÉROS DES PAGES DANS L'ORIGINAL
not given	YEAR / ANNÉE	VOLUME	ISSUE NO. / NUMÉRO	217-227
PLACE OF PUBLICATION / LIEU DE PUBLICATION ---	1977	110	-	NUMBER OF TYPED PAGES / NOMBRE DE PAGES DACTYLOGRAPHIÉES: 16

REQUESTING DEPARTMENT / MINISTÈRE-CLIENT: Environment

BRANCH OR DIVISION / DIRECTION OU DIVISION: Fisheries

PERSON REQUESTING / DEMANDÉ PAR: Dr. Kovacs

YOUR NUMBER / VOTRE DOSSIER N°: 1448477

DATE OF REQUEST / DATE DE LA DEMANDE: August 25, 1977

TRANSLATION BUREAU NO. / NOTRE DOSSIER N°: 1448477

TRANSLATOR (INITIALS) / TRADUCTEUR (INITIALES): MMA

OCT 4 1977

UNEDITED TRANSLATION
For Information only
TRADUCTION NON REVISEE
Information seulement

SOS-200-10-6 (REV. 2/68)
7530-21-029-5333

Fdm#4128

Secretary of State / Secrétariat d'État

TRANSLATION BUREAU / BUREAU DES TRADUCTIONS

MULTILINGUAL SERVICES DIVISION / DIVISION DES SERVICES MULTILINGUES

CLIENT'S NO. / N° DU CLIENT	DEPARTMENT / MINISTÈRE	DIVISION/BRANCH / DIVISION/DIRECTION	CITY / VILLE
1448477	Environment	Fisheries	Ottawa
BUREAU NO. / N° DU BUREAU	**LANGUAGE / LANGUE**	**TRANSLATOR (INITIALS) / TRADUCTEUR (INITIALES)**	
1448477	German	MMA	OCT 4 1977

LUDWIG, Helga, REINER, J. and SPITELLER, G.:
Untersuchung der Steroide im Blut mit der Kombination Glaskapillargaschromatographie-Massenspektrometrie.

[Investigation of the steroids in blood with the combination glass capillary column gas chromatography - mass spectrometry].

Chem. Ber. 110: 217-227 (1977).

SUMMARY

Glass capillary column gas chromatography-mass spectrometry was applied for the investigation of steroids in blood samples. Ten so far as components of blood unknown steroids were detected and characterized: 3α-hydroxy-5-androsten-17-one, 3,17-dihydroxy-5-androsten-7-one, 3,7-dihydroxy-5-androsten-17-one, 6,17-dihydroxy-4-androsten-3-one, 3,7,17-trihydroxy-5-androstene (two isomers), 3,16,17-trihydroxy-androstane (three isomers), and 3,16,20-trihydroxy-5-pregnene.

Organisch-Chemisches Institut
of the University of Göttingen
Tammannstrasse 2, D-3400 Göttingen

Received: April 21, 1976.

Currently steroids in the blood are usually determined by radio-isotope analysis although - unfortunately - only isolated steroids can be detected by this method (1). But, in many instances a quantitative analysis of as many steroids as possible, if not all the steroids present in blood, would be most desirable as their relations to one another might be equally important.

Simultaneous detection of all steroids contained in the blood is extremely difficult: with the exception of cholesterol, all other steroids are only represented by trace amounts. Cholesterol, fatty acids, fats andproteins, quantitatively exceeding total concentrations of all other steroids several thousand-fold, must be separated almost completely from the rest. In this process traces of some steroids might be lost.

We are indebted to Sjővall and his colleagues for preliminary investigations on blood steroids (2,3). This group first fractionated the steroid conjugates present in the blood into glucuronides, monosulfates and disulfates, subsequently saponified the conjugates, and finally separated the methylsilylated steroids by gas chromatography on a packed column, and then identified them by mass spectrometry. This method has the disadvantage that steroids with similar retention times can no longer be separated; it is therefore impossible to elicit unequivocal estimates of trace steroids.

Recently methods of gas chromatographic separation have been improved considerably through the use of capillary columns (4). Now we have applied this method for the first time to the 218
investigation of blood steroids and the preparation of suitable steroid profiles. The method has made it possible to identify a number of steroids not previously found in blood. As the method is generally also suitable for semi-quantitative steroid assay - with the sole exception of corticoids - it is now possible to determine differences in blood steroid levels in healthy persons and patients.

Blood plasma was worked up by a modified method based on that of Sjővall. Since - with the exception of cholesterol - the major portion of steroids is present in the form of sulfates,

preference was given to the investigation of sulfate fractions. The separation process was checked for reproducibility and recovery rate with a radioactive steroid, [7-^{3}H]-dehydroepiandrosterone sulfate ammonium salt, and modified accordingly.

First of all we were able to note that at the beginning of the work procedure approximately 60% of the radioactive steroid are already lost when proteins are precipitated (2) with organic solvents. But, the recovery rate can be raised to approximately 90% when the plasma is diluted ten-fold with physiological saline to prevent the precipitation of proteins (5), and steroids are subsequently separated from the proteins and salts, together with accompanying secondary substances, such as cholesterol, fats and fatty acids, on XAD-4 (6,7). Although it is thus possible to isolate the monosulfate fraction with only minor losses, the disulfates - and thus a considerable portion of the corticosteroids - are lost when the XAD-4 is washed with water (8).

Subsequently the steroid conjugates were fractionated into glucuronides, monosulfates and disulfates on Sephadex-LH-20 (2), and afterwards the sulfate fractions were saponified enzymatically with helicase (9). However, this reaction does not proceed quantitatively: therefore it requires after-saponification according to the method of Burstein and Liberman (10). In most instances the fractions obtained by acid and enzymatic saponification still contained substantial amounts of fatty acids which either had not been separated out on Sephadex or may have originated from acid and enzymatically saponified glycerides (commercially available helicase contains small amounts of esterase). The free fatty acids could be separated quantitatively on DEAP-LH-20 (11,12), a weakly basic ion exchanger on a Sephadex base. The steroid fraction which underwent acid saponification still contained a great deal of cholesterol which was removed on Lipidex$^{(R)}$-5000.

Subsequently the free steroids of the three fractions were freed on nonpolar secondary substances on silica gel. As free steroids are adsorbed too intensely by gas chromatographic columns and tend to separate poorly, they were reacted with 219
2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)acetamide to give trimethylsilyl ethers before they were analyzed by combination

glass capillary column gas chromatography - mass spectrometry, and finally identified by their mass spectra.

Preparation of profiles: After measuring in the combination glass capillary column gas chromatography - mass spectrometer, a second glass capillary column gas chromatogram was taken from each fraction to provide at least semi-quantitative data. Quantitative determination of steroids in glass capillary columns is highly problematic: fluctuations of up to 20% are constantly encountered.

Characterization by retention indices: In a third glass capillary column chromatogram hydrocarbons were added to determine retention indices, because in many isomeric compounds only a combination of mass spectra and retention indices will permit unequivocal identification.

Quite frequently it is possible to determine the positions of the hydroxyl groups in the ring system from the mass spectra, but not their configuration. As the retention times of steroids, whose hydroxyl groups occupy the same positions though they exhibit different configurations, differ noticeably in the glass capillary column gas chromatogram (13,14), steroid trimethyl silyl ethers of a known structure were added to the steroid mixture isolated from the blood.

Simultaneous injection with steroid silyl ethers has the advantage that evaluation of the chromatograms is independent of the almost unavoidable measuring fluctuations. A disadvantage of this method is that measurements must be repeated frequently to facilitate unequivocal steroid classification in the various glass capillary column gas chromatograms. The retention indices of the steroid-trimethylsilyl ethers were related to the unbranched hydrocarbons $C_{24}H_{50}$ and $C_{32}H_{66}$. The error in the determination of retention indices is approximately 0.5%.

RESULTS

1. Monosulfate fractions:

a). The glass capillary column gas chromatogram depicted in Fig. 1 illustrates an enzymatically saponified monosulfate fraction obtained from 1 liter of plasma of male and female donors.

The running variable numbers added to the peaks shown in the glass capillary column chromatograms correspond to the steroids listed in Table 1. Retention indices of the steroid trimethylsilyl ethers and the compounds used for comparison are also given. (Compounds with the same running variable (without index a,b,c...) correspond to the same steroids in all gas chromatograms and are therefore not listed in every Table.

Mass spectra taken from steroids with a very low con- 220
centration in the mixture (corresponding to very small gas chromatogram peaks, e.g. that of 3,16,20-trihydroxy-5-pregnene, a compound so far not known as a blood component) are usually of very feeble intensity. Their identification is complicated by traces of secondary substances or column material present in the mixture. Quite often spectra are obtained which suggest that they hail from a mixture of equivalent amounts of several compounds. In such cases, conclusions on structure can often only be drawn by measuring the retention indices of the steroid trimethylsilyl ethers.

The possibilities and limitations in detecting steroid traces by the combined glass capillary column gas chromatography - mass spectrometry method shall be demonstrated on the example of the mass spectrum reproduced in Fig. 2:

The spectrum would seem to suggest that one is dealing with a compound of molecular mass 548. But, the presence of a peak of mass 460 shows unequivocally that the major component has the mass 550 because splitting off of a particle of 88 mass units is not feasible in steroid-trimethylsilyl ethers. Therefore, the peak of mass 548 must hail from some admixture, the peaks marked with an asterisk may also be classified as contaminants.

This shows clearly that not even with glass capillary columns packed with SE 30 is it always possible to separate compounds with almost identical retention times and barely differentiated molecular mass. Yet, the preparation of columns with polar phases, such as OV 17 for example, is still rather difficult.

The molecular mass suggests a trihydroxypregnene. 222
The problem that confronts one is localizing the functional groups:

The peak at m/e = 117 points to pregnene which has a trimethylsilylated hydroxyl group in position 20 (15). It

Fig. 1: Glass capillary column gas chromatogram of the steroid monosulfate fraction from 1 liter of blood plasma of various persons (for details, see experimental section).

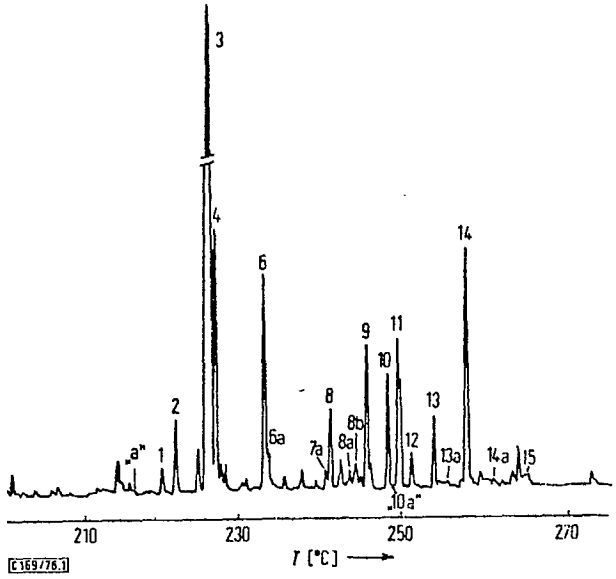

Table 1: Retention indices of the steroid-trimethylsilyl ethers separated in the chromatogram in Fig. 1, and corresponding control substances.

Nr. = #
Name = name
Masse = mass
des = of der
Vergleichs-verbindung = of the control substance

*These steroids were identified by GC additionally through simultaneous injection of corresponding TMS ethers.

Nr.	Name	Mol.-Masse des TMS-Ethers	Retentionsindex (SE 30)	Retentionsindex der Vergleichsverbindung (SE 30)
„a"	3α-Hydroxy-5-androsten-17-on	360	2428	2428
1	3α-Hydroxy-5α-androstan-17-on	362	2446	
2	3α-Hydroxy-5β-androstan-17-on	362	2472	
3	3β-Hydroxy-5-androsten-17-on	360	2526	2525
4	3β-Hydroxy-5α-androstan-17-on	362	2544	
6	3β,17β-Dihydroxy-5-androsten	434	2632	
6a	3β,17β-Dihydroxy-5α-androstan	436	2638	2638
7a	3,16-Dihydroxy-5-androsten-17-on	448	2748	
8	3β,16α-Dihydroxy-5-androsten-17-on*)	448	2762	2764
8a	Hydroxyandrostendion	374		
8b	3.20-Dihydroxy-5-pregnen-on	550		
9	3,16,17-Trihydroxy-5-androsten	522		
10	3β,20α-Dihydroxy-5-pregnen*)	462	2858	2860
„10a"	3,16,17-Trihydroxy-androstan	524	2867	
11	3β,16α,17β-Trihydroxy-5-androsten*)	522	2878	2880
12	3,16,20-Trihydroxy-5-pregnen	550	2902	
13	3β,17α,20α-Trihydroxy-pregnan	480	2948	
13a	6(?),20-Dihydroxy-4-pregnen-3-on	476		
14	3β,17α,20α-Trihydroxy-5-pregnen*)	478	3017	3020
14a	x,x,20-Trihydroxy-pregnen-on	494		
15	3β-Hydroxy-5-cholesten	458	3134	

corresponds to C-atoms 20 and 21. The two peaks at m/e = 156 and 157 are characteristic for a trimethylsilylated 16,20-dihydroxy-pregnane structure and correspond to C-atoms 15, 16, 17, 20 and 21 with a trimethylsilylated hydroxyl group in position 20 under splitting off of the functional group in position 16 (16). This conclusion is further strengthened by a peak of mass 141, corresponding to the loss of CH_3 from the ion of mass 156.

The peak at m/e = 129 argues for a steroid which - in position 3 - has a trimethylsilylated hydroxyl group and a Δ^5-double bond (17). The remaining peaks in the spectrum obviously originate from admixtures.

No trace steroids can be discovered on recording glass capillary column gas chromatograms like that illustrated in Fig. 1 because the peaks are not sufficiently intensive. Nevertheless, they can be demonstrated and a partial structure can be derived, either by concentrating the trace steroid sufficiently, which is an expensive process, or by charging the columns with high doses of the complete mixture so that even steroid traces will still yield identifiable mass spectra. Using the latter method, it was possible to identify a 3,16,17-trihydroxy-androstane with a retention index of I = 2867 in the monosulfate fraction described above. In the glass capillary column gas chromatogram (Fig. 1) this compound is no longer indicated by a peak because its concentration is too low by comparison with the major components. According to its retention index this steroid would have to be inserted between peaks 10 and 11.

The mass spectrum (Fig. 3) shows a triple trimethylsilanol splitting, followed by elimination of CH_3. The appearance of ions of mass 147 and 191 is characteristic for compounds with vicinal trimethylsilyl groups (18). 3,16,17-trihydroxyandrostane, recently isolated by our colleague, H. Grote, shows considerable 223 agreement between its TMS ether spectra and the spectrum reproduced in Fig. 3: all contain an ion of mass 318, equivalent to the loss of C-16 and C-17, as well as an ion of mass 305, which indicates the splitting off of C-atoms 15 - 17, and disintegrates further to a fragment of mass 215 due to the loss of the trimethyl-

Fig. 2: Mass spectrum of 3,16,20-trihydroxy-5-pregnene-tris(trimethylsilyl ether).

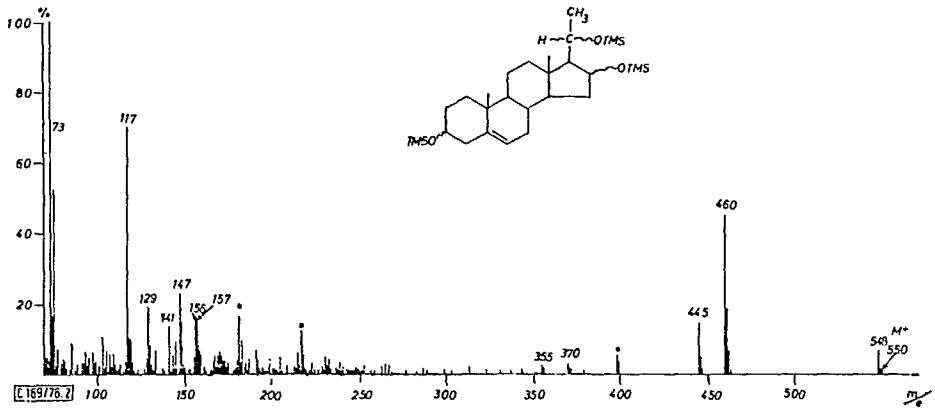

Fig. 3: Mass spectrum of 3,16,17-trihydroxyandrostane-tris(trimethylsilyl ether)

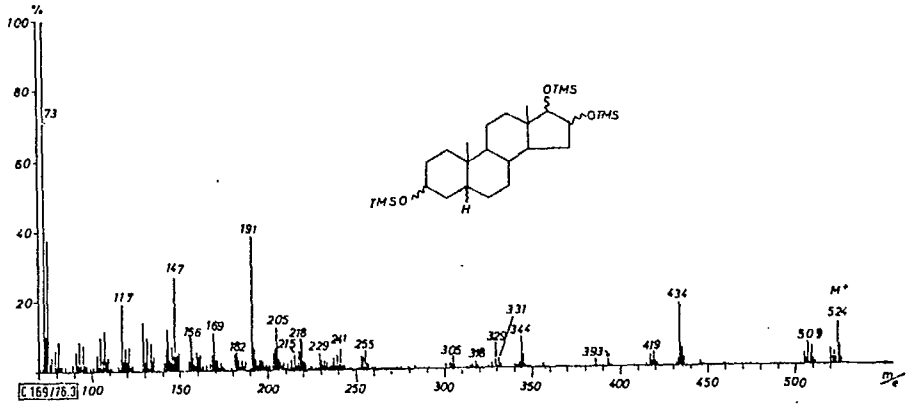

Table 2: Retention indices of 3,16,17-trihydroxyandrostanes and hydroxyandrostenones.

3,16,17-Trihydroxyandrostan	Retent.-Index (SE 30)	Hydroxyandrostenon	Retent.-Index (SE 30)
ααβ-5α	2800	3α-Hydroxy-5-androsten-17-on	2428
βαβ-5α	2892	3β-Hydroxy-5-androsten-17-on	2525
βββ-5β	2712	17α-Hydroxy-4-androsten-3-on	2593
βαβ-5β	2778	17β-Hydroxy-4-androsten-3-on	2643
ααβ-5β	2788	Peak „a"	2428
αββ-5β	2830		
Peak „10a"	2867		

Fig. 4: Glass capillary column gas chromatogram of the steroid-trimethylsilyl ethers of the enzymatically saponified steroid monosulfate fraction from 40 ml of plasma of a woman.

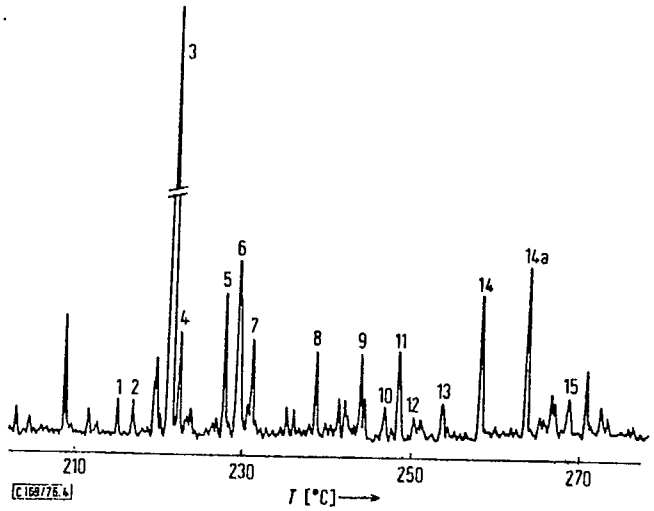

Table 3: Steroids identified additionally in the isolated sample depicted in Fig. 4, by comparison with pooled plasma (Fig. 1).

Nr.	Name	Mol.-Masse des TMS-Ethers	Retentionsindex (SE 30)	Retentionsindex der Vergleichsverbindung (SE 30)
5	3β-Hydroxy-5-androsten-17-on (Enol)	432	2610	
7	3β-Hydroxy-5α-androstan-11,17-dion	448	2654	2652
14a	(unidentifiziertes Pregnanderivat)		3074	

Vergleichsverbindung = control compound
unidentifiziertes Pregnanderivat = unidentified pregnane derivative

Table 4: Retention indices of the steroid trimethylsilyl ethers separated in the chromatogram depicted in Fig. 5 and corresponding control compounds.

Nr.	Name	Mol.-Masse	Retentionsindex (SE-30)	Retentionsindex des Vergleichssteroids
6a	Dihydroxy-4-androsten-3-on	448	2654	
6b	3,16,17-Trihydroxyandrostan	524	2668	
8a	3,16,17-Trihydroxyandrostan	524	2798	2800
P	Dioctylphthalat			

Vergleichs-steroid = control steroid used for comparison

silyl group in position 3. The ion of mass 331 which evolves from the splitting off of C-17 and two trimethylsilyl ether groups is also characteristic for such compounds. It further disintegrates - through loss of trimethylsilanol - to an ion of mass 241.

Isomers can only be differentiated by measuring retention indices. In our case it was impossible to determine the configuration of the 3,16,17-trihydroxyandrostane.

A further new blood component identified was 3α-hydroxy-5-androsten -17-one (peak "a"). As the spectra of the trimethylsilyl ethers of theisomers 3β-hydroxy-5-androsten -17-one (DHEA) 17β-hydroxy-4-androsten -3-one (testosterone), 17α-hydroxy- 224
4-androsten -3-one (epitestosterone) and 3α-hydroxy=5-androsten -17-one are very similar, in these cases too retention indices had to be determined for identification.

b). In Fig. 4 the glass capillary column gas chromatogram of pooled plasma has been compared with the chromatogram of an isolated sample of 40 ml of plasma from a woman. The steroids of the plasma pool and the isolated sample differ considerably. Routine investigations are required to correlate these differences with certain physical conditions. It is probable that - following the necessary improvements of work procedures - in future these differences will be used to diagnose diseases in steroid metabolism. Our colleague, H. Egger, was already able to do this successfully with respect to hirsutism; a report on this work will be found elsewhere (18a).

The steroids additionally identified in the isolated plasma sample are summarized in Table 3.

2). Disulfate fractions:

Withrespect to their qualitative composition, the gas chromatograms of the disulfate fractions resemble those of the monosulfate fractions, especially in view of the fact that monosulfates are frequently carried over into the disulfate fractions.

The steroids identified are listed in Table 4. Two further isomers of 3,16,17-trihydroxy-androstane were unknown before; according to its retention index, one of these may be identical with 3α,16α,17β-trihydroxy-5α-androstene.

Fig. 5: Steroid disulfate fraction from 250 ml of a man's plasma.

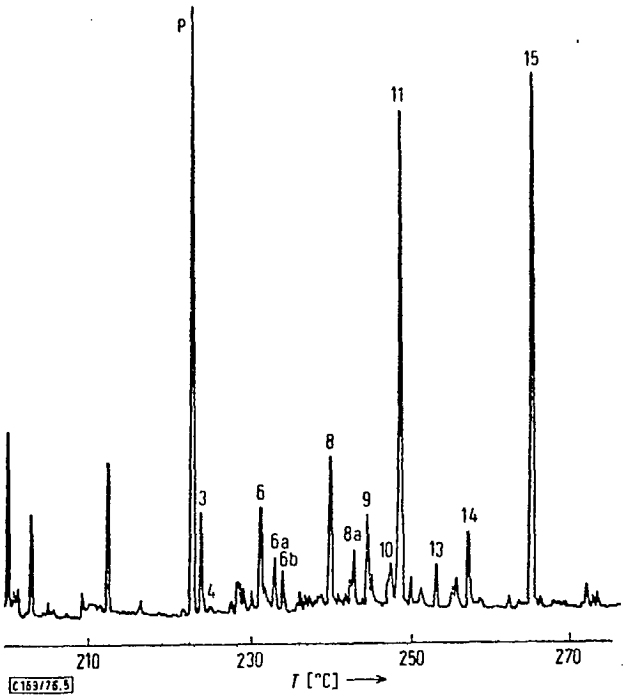

Table 5: Retention indices of steroids (as TMS ethers) so far unknown as blood components.

Name	Mol.-Masse	Retentions-index (SE 30)
3,7-Dihydroxy-5-androsten-17-on *)	448	2614
3,7-Dihydroxy-5-androsten-17-on *)	448	2672
3,17-Dihydroxy-5-androsten-7-on	448	2871
6,17-Dihydroxy-4-androsten-3-on	448	2708
3,7,17-Trihydroxy-5-androsten	522	2656
3,7,17-Trihydroxy-5-androsten	522	2794

*3β,7α-dihydroxy-5-androsten-17-one (19) is known to be a blood component.

3). Further new blood steroids:

Five further steroids - so far not known as blood components - were identified by combined glass capillary column gas chromatography - mass spectrometry (Table 5) in the course of this work.

226

We wish to thank the 'Deutsche Forschungsgemeinschaft' as well as the 'Fonds der Chemischen Industrie' for supporting this work through material aid. A further part of this work was supported by funds from the 'Toto-Lotto' for which we thank Lower Saxony. Finally, we thank the LKB Company in Stockholm for lending us the LKB-mass spectrometer with computer.

****

EXPERIMENTAL SECTION

I. Instruments used:

a). Separation of steroid-trimethylsilyl ethers and preparation of mass spectra was done with an LKB 2091-mass spectrometer and LKB 2130-data system (with PDP-11 computer), ionization energy 70 eV. The TIC was recorded at 20 eV. 20 m glass capillary columns (internal diameter 0.3 mm, SE-30-thin film), 2 ml He/min., injector temperature: 275°C, column temperature: 120 - 300°C, programmed: 3°C/min.

b). Glass capillary gas chromatograms and retention indices of TMS ethers were measured on a Carlo Erba 2300 gas chromatograph. Detector: FID, injector temperature: 275°C, temperature program 120 - 300°C, 2°C/min. Thin film capillary column (20 m, 0.3 mm internal diameter) charged according to statistical method (20). Alkali glass (Hilgenberg-glass, Malsfeld) was used. Capillary column pull instrument: Hupe and Busch.

c). Scintillations were measured with a Tri-Carb 3380 liquid scintillation counter, Packard, duration of counting: 10 min. Scintillator fluid: 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) dissolved in toluene/ethanol.

II. Isolation of steroid sulfates from blood plasma: 1 liter of plasma was diluted to 10 liters with physiological saline (5) and poured over an XAD-4 column (300 g) (Serva, Heidelberg) at a flow rate of 100 ml/hr. The column material was washed with

2 liters of water and the adsorbed substances eluted with 1 liter of methanol. The methanol extract dried in a vacuum was separated into three fractions on Sephadex-LH-20 (4 G) (Pharmacia Chemicals, Uppsala) with the solvent system methanol/chloroform (1:1) to which 0.01 mol NaCl had been added; flow rate: 4 ml/hr. The steroid glucuronnides and accompanying substances were eluted in a volume of 0 - 29 ml, the steroid monosulfates from 30 - 65 ml, the disulfates from 66 - 250 ml. 20 ml of acetate buffer, adjusted to pH 4.7 was added to the monosulfate fraction as well as the disulfate fraction; then they were saponified with 0.2 ml of a solution of β-glucuronidase/arylsulfate (Boehringer, Mannheim) for three days at 37°C. The free steroids were isolated by extracting three times, each time with 50 ml of ethyl acetate. The aqueous phases of the ethyl acetate extractions were combined and passed once more through an XAD-4 column (100 g) to isolate the unsaponified sulfates. Following desalting (200 ml of water) the mixture was eluted with 100 ml of methanol, and the residue treated with H_2SO_4-acidified ethyl acetate (20 ml) for 24 hrs. at 39°C, according to Burstein and Lieberman (10). Following neutralization of the sulfuric acid with $NaHCO_3$, the ethyl acetate was eliminated in a vacuum.

All saponified fractions were freed of fatty acids on DEAP-LH-20 (8g)(prepared from Sephadex-LH-20)(11) with the solvent system methanol/chloroform/water (9:2:1), at a flow rate of 4 ml/hr. (12).

The same solvent system was used to separate cholesterol from the steroid mixtures which had undergone acid saponification, on Lipidex$^{(R)}$-5000 (5 g)(Packard, Zurich); flow rate: 5 ml/hr.

Each sample was applied to a benzene slurried silica gel column (200 mg) in the solvent mixture system benzene/ethyl acetate (95:5) (21). It was possible to remove nonpolar compounds 227
with 30 ml of this solvent. After the solvent had been changed, polar substances were extracted with 30 ml of ethyl acetate. The ethyl acetate extracts were dissolved in 10 µl of methanol; of this - following trimethylsilylation - two thirds were used for mass spectrometric analysis of the GC-MS coupling, and one third was used to prepare glass capillary column gas chromatograms.

III. Checking the work up procedure with [7-^{3}H] dehydroepiandrosterone sulfate, ammonium salt:

To each aliquot of 100 ml of plasma were added 0.05 µCi [7-^{3}H]-dehydroepiandrosterone sulfate ammonium salt (corresponding to 111400 Ipm)(NEN New England Nuclear Chemicals, Frankfurt) and 30 µg of unlabeled dehydroepiandrosterone sulfate. The work up procedure was performed three times with the trit. steroid. After each separating step, residual radioactivity was determined with a liquid scintillation counter. Thus, three measuring values were obtained for each working step, then the mean of these three values was calculated.

Adsorption and subsequent elution of the radioactive steroid on XAD-4 proceeded with a 90% recovery rate; on the other hand, upon protein precipitation with organic solvents, the recovery rate was only 40%. 85% of the radioactivity still present following separation of protein, could be determined after Sephadex separation; a further 75% of residual activity after enzymatic saponification and subsequent extraction of the labeled steroid. Separation of fatty acids on DEAP-LH-20 yielded a recovery rate of 85%, and the elimination of nonpolar compounds on silica gel on the average 70%.

Although each separation step proceeded with a recovery rate of 70 - 90%, approximately 65% of the radioactivity employed were lost during the 5-step separation process. When the work procedure was simulated with a purely radioactive substance without blood plasma, the recovery rate after each separation step was 10% higher on the average. We assume that the loss of labeled steroid during separation of mixtures containing plasma is attributable to blood components of high concentration which interfere considerably with the recovery of tritium labeled DHEA.

IV. Preparation of trimethylsilyl ethers: The samples were taken up with methanol, put into small test tubes, the solvent removed, and then 3 µl of 2,2,2-trifluoro-N-methyl-N-(trimethylsilyl)acetamide (Macherey and Nagel, Düren) were added. The test tubes were sealed by fusion and then left at room temperature for 24 hrs. The reaction mixture was then examined directly in the combined GC-MS without any further preparatory work.

****

REFERENCES

[1] *G. E. Abraham*, Anal. Lett. **5**, 915 (1972).
[2] *J. Sjövall, K. Sjövall* und *R. Vihko*, Steroids **11**, 703 (1968).
[3] *O. Jänne, R. Vihko, J. Sjövall* und *K. Sjövall*, Clin. Chim. Acta **23**, 405 (1969).

[4] *D. Henneberg, U. Henrichs* und *G. Schomburg*, J. Chromatogr. **112**, 343 (1969); *G. Alexander* und *G. A. F. M Rutten*, Chromatographia **6**, 231 (1973); J. Chromatogr. **99**, 81 (1974); *G. Schomburg, H. Husmann* und *F. Weeke*, J. Chromatogr. **99**, 63 (1974); *K. Grob*, Chromatographia **7**, 94 (1974).
[5] *H. Dengler*, persönliche Mitteil.
[6] *H. L. Bradlow*, Steroids **11**, 265 (1968).
[7] *G. Machata* und *W. Vycudilik*, Arch. Toxicol. **33**, 115 (1975).
[8] *M. Matsui, M. Hakozaki* und *Y. Kinuyama*, J. Chromatogr. **115**, 625 (1975).
[9] *C. G. Beling*, Acta Endocrinol. (Copenhagen), Suppl. **79**, 46 (1963).
[10] *S. Burstein* und *S. Lieberman*, J. Biol. Chem. **233**, 331 (1958).
[11] *B. Almé* und *E. Nyström*, J. Chromatogr. **59**, 45 (1971).
[12] *M. Axelson* und *J. Sjövall*, J. Steroid Biochem. **5**, 1 (1974).

[13] *K. B. Eik-Nes* und *E. C. Horning*, Gas Phase Chromatography of Steroids, Springer Verlag, Berlin/Heidelberg/New York 1968.
[14] *J. A. Luyten* und *G. A. F. M. Rutten*, J. Chromatogr. **91**, 393 (1974).

[15] *J. Sjövall* und *R. Vihko*, Steroids **7**, 447 (1966).
[16] *J. A. Gustafsson, C. H. L. Shackleton* und *J. Sjövall*, Eur. J. Biochem. **10**, 302 (1969).
[17] *C. W. Brooks*, Org. Mass Spectrom. **7**, 925 (1973).
[18] *J. A. Gustafsson, R. Ryhage, J. Sjövall* und *R. M. Moriarty*, J. Amer. Chem. Soc. **91**, 1234 (1969).

[18a] *H.-J. Egger, J. Reiner, G. Spiteller* und *R. Häffele*, Clin. Chim. Acta (im Druck). (in press).

[19] *L. Starka* und *R. Hampe*, Naturwissenschaften **51**, 164 (1964).

[20] *J. Bouche* und *M. Verzele*, J. Chromatogr. **6**, 501 (1968).

[21] *R. Vihko* und *O. Jänne* in Gaschromatography of Hormone Steroids (*R. Scholler*, Paris 1967, Proceedings of the Round Table Conference).