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THE GAS CHROMATOGRAPHY OF AROMATIC ACIDS AS THEIR TRIMETHYLSILYL DERIVATIVES, INCLUDING APPLICATIONS TO URINE ANALYSIS

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SUMMARY

The preparation of trimethylsilyl derivatives from aromatic acids and a few related lactones was examined under various conditions. Indoles and amides frequently gave more than one derivative, particularly with bis-(trimethylsilyl)-acetamide; the formation and stability of derivatives were also influenced by time, solvent and the presence of water or of substances possessing catalytic activity. The most suitable silylating reagent examined appeared to be hexamethyldisilazane in the presence of trifluoroacetate.

Methylene unit values on OV-17 and OV-1 were recorded for derivatives of 79 compounds, mostly acidic, containing a phenyl or indolyl nucleus together with various combinations of unsaturated, amide, methoxyl and phenolic, alcoholic or enolic hydroxyl groups. Values were higher on OV-17 to an extent determined by the number of hydroxyl, methoxyl and especially NH groups in a molecule. Small variations on a given liquid phase with experimental conditions appeared to be related largely to the number of hydroxyl groups in a molecule.

With urine extracts, quantitative results satisfactory for many purposes were obtained for 4-hydroxy-3-methoxymandelic acid on OV-17 and for *p*-hydroxyphenyllactic acid on OV-1 or, less satisfactorily, OV-17.

INTRODUCTION

Since chromatographic techniques first began to be applied to urine analysis no group of urinary constituents has attracted more interest than the aromatic acids. Phenols and indoles, both easily detected by paper chromatography, have received most attention but gas chromatographic methods are now beginning to facilitate studies in a wider field.

The extraction of aromatic acids from urine and their gas chromatography as trimethylsilyl derivatives have been the partial subjects of previous publications[1–4]. However the chromatographic behaviour of only a limited number of authentic substances has been recorded as yet. Moreover, although the simpler compounds appear to undergo trimethylsilylation at carboxyl and hydroxyl groups with reagents such as bis-(trimethylsilyl)-acetamide or hexamethyl-disilazane, discrepancies in the re-

corded[2,3] behaviour of compounds containing the NH group appear most readily to be explained in terms of the silylation process.

More detailed investigations into the behaviour of aromatic acids form the subject of this paper.

EXPERIMENTAL

Definitions and abbreviations

The term silylation is used rather than the more accurate trimethylsilylation; derivatives are referred to as TMS derivatives. The following abbreviations are also used: BSA, bis-(trimethylsilyl)-acetamide; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; TFA, trifluoroacetic acid; pyr, pyridine. Benzoylglycine and its derivatives are referred to as hippuric acids and phenylacetylglycine and its derivatives as phenaceturic acids. Two physiologically important phenolic acids, 4-hydroxy-3-methoxymandelic acid and *p*-hydroxyphenyllactic acid, are referred to as VMA and PHPL respectively.

The chromatographic properties of each substance, described in terms of methylene unit (MU) values, was determined by the procedure of DALGLIESH *et al.*[2] using as standards even-numbered straight-chain hydrocarbons from C_{12} to C_{28}. Δ MU (OV-17 — OV-1) values were obtained by subtracting values found using long OV-1 columns from those using long OV-17 columns and δMU (OV-17 or OV-1) values by subtracting values obtained using the short from those using the long columns.

The chromatographic behaviour of some amides and indoles was complicated by their tendency to form two or more peaks. In practice the peak (or major peak) formed using HMDS (Table II) was considered to be normal and additional peaks, all obviously formed more readily with BSA (Tables I and II) were considered to be "difficult".

Materials

All reagents were of the best available quality. Pyridine was stored over solid KOH. BSA and reagents derived from it were prepared from freshly opened 1 ml ampoules (Sigma London Ltd.) and used immediately. The following silylating reagents were employed: BSA; BSA–pyr (1:1); BSA–TMCS (4:1); HMDS–pyr (no fixed composition; sometimes TMCS was added as catalyst); HMDS–TFA prepared by adding TFA (0.5 ml) and HMDS (4.5 ml) to pyridine (5 ml); the mixture was allowed to stand overnight and appeared to be stable indefinitely if protected from moisture.

Most aromatic acids were obtained commercially but many were synthesised or received as gifts for which we are particularly indebted to Dr. J. W. T. SEAKINS. Most substances appeared to be chromatographically pure although small secondary peaks were occasionally observed. A few of the cheaper commercial products such as hippuric, cinnamic and 2,6-dihydroxybenzoic acids gave appreciable secondary peaks. Several aroylamides gave peaks identified as the parent aromatic acids, of whose possible presence we were warned. Minor peaks from ketoacids were probably decomposition products of these unstable compounds. A sample of *m*-hydroxyhippuric acid isolated from the urine of a subject fed *m*-hydroxybenzoic acid contained hippuric acid as impurity. Since no authentic sample was available, *m*-hydroxyphenylhydracrylic acid, a major constituent, was isolated from human urine by paper chromatography.

TABLE I

METHYLENE UNIT VALUES OF AROMATIC ACIDS SILYLATED WITH BSA

Correlations of peaks on OV-1 with those on OV-17 were not attempted where ambiguity was evident.

Acid	*Column*				
	OV-17 (1.5m)	*OV-1 (1.5m)*	*OV-17*	*OV-1*	*Δ MU (OV-17 – OV-1)*
Benzoic	13.65	12.26	13.96	12.31	1.65
Hippuric[a]	21.05	18.02	21.13	18.04	3.09
Phenylacetic	14.40	12.64	14.40	12.72	1.68
Phenaceturic	21.67	18.47	21.77	18.43	
		18.62		18.57	
Phenylacetylglutamic	24.68	20.67	24.91	20.68	
	26.06	22.39	26.00	22.35	
		22.96		22.95	
Phenylacetylglutamine[a]	24.67	20.68	24.93	20.68	
	24.97	20.80	25.21	20.82	
	27.96	21.95	*ca.* 28.4	22.00	
			ca. 28.5		
β-Phenylpropionic	15.52	14.05	15.65	14.06	1.59
Cinnamic	17.04	14.97	17.17	15.21	1.96
Mandelic	15.91	14.69	15.90	14.57	1.33
β-Phenyllactic[a]	16.88	15.87	16.83	15.82	1.01
Phenylpyruvic[a]	18.28	17.02	18.32	16.99	1.33
N-Acetylphenylalanine[a]	19.39	17.88	19.31	17.90	
	20.54		20.65		
o-Hydroxybenzoic[a]	16.26	15.01	16.30	15.00	1.30
o-Hydroxyhippuric[a]	22.92	20.41	23.02	20.51	2.51
o-Hydroxyphenylacetic[a]	16.92	15.56	16.85	15.53	1.32
o-Hydroxyphenylpyruvic, lactone	18.95	16.50	19.32	16.74	2.58
o-Hydroxycinnamic	19.57	18.00	19.62	18.03	1.59
m-Hydroxybenzoic[a]	16.66	15.57	16.76	15.51	1.25
m-Hydroxyhippuric[a]	23.80	21.20	23.78	21.20	2.58
m-Hydroxyphenylacetic[a]	17.36	15.97	17.25	15.97	1.28
β-(*m*-Hydroxyphenyl)propionic[a]	18.52	17.17	18.48	17.13	1.35
m-Hydroxycinnamic	20.05	18.63	20.00	18.57	1.43
β-(*m*-Hydroxyphenyl)hydracrylic[a]	19.41	18.63	19.22	18.50	0.72
p-Hydroxybenzoic[a]	17.32	16.20	17.25	16.20	1.05
p-Hydroxyphenylacetic[a]	17.65	16.29	17.59	16.25	1.34
β-(*p*-Hydroxyphenyl)propionic[a]	18.88	17.53	18.83	17.48	1.35
p-Hydroxycinnamic[a]	20.84	19.28	20.84	19.30	1.54
p-Hydroxymandelic[a]	18.88	17.94	18.65	17.74	0.91
β-(*p*-Hydroxyphenyl)lactic[a]	20.00	19.13	19.86	19.05	0.81
p-Hydroxyphenylpyruvic[a]	21.59	20.61	21.49	20.54	0.95
N-Acetyltyrosine[a]	24.86	21.29	24.90	21.24	3.66
p-Methoxybenzoic	16.96	14.95	17.01	15.04	1.97
p-Methoxyhippuric	24.47	20.79	24.58	20.83	3.75
p-Methoxyphenylacetic	17.28	15.13	17.33	15.10	2.23
p-Methoxyphenaceturic	24.75	20.82	24.82	20.85	3.97
p-Methoxyphenylacetylglutamic	27.80	23.19	*ca.* 28.1	23.16	
	ca. 28.8	24.44	*ca.* 28.8	24.42	
β-(*p*-Methoxyphenyl)propionic	18.46	16.30	18.54	16.36	2.18
p-Methoxycinnamic	20.58	17.96	20.69	18.08	2.61
p-Methoxymandelic	18.75	16.91	18.64	16.81	1.83
β-(*p*-Methoxyphenyl)lactic	19.62	17.95	19.60	18.01	1.59
p-Methoxyphenylpyruvic	21.42	19.54	21.46	19.54	1.92
N-Acetyl-*p*-Methoxyphenylalanine	23.51	20.09	23.56	20.13	3.43

TABLE I (*continued*)

Acid		Column				
		OV-17 (1.5m)	*OV-1 (1.5m)*	*OV-17*	*OV-1*	*Δ MU- (OV-17 - OV-1)*
2,3-Dihydroxybenzoic		18.48	17.49	18.45	17.51	0.94
2,4-Dihydroxybenzoic		19.20	18.23	19.07	18.18	0.89
2,5-Dihydroxybenzoic[a]		18.78	17.85	18.60	17.74	0.86
2,5-Dihydroxyphenylacetic[a]		19.40	18.45	19.27	18.32	0.95
2,5-Dihydroxyphenylacetic, lactone[a]		18.70	17.30	18.71	17.30	1.41
2,5-Dihydroxyphenylpyruvic, lactone[a]		22.03	20.21	22.27	20.35	1.92
2,6-Dihydroxybenzoic		18.86	17.68	18.81	17.64	1.17
3,4-Dihydroxybenzoic[a]		19.17	18.25	19.05	18.21	0.84
3,4-Dihydroxyphenylacetic[a]		19.39	18.35	19.31	18.30	1.01
3,4-Dihydroxyphenylcinnamic[a]		22.48	21.49	22.43	21.40	1.03
3,4-Dihydroxymandelic[a]		20.19	19.48	20.02	19.33	0.69
3,5-Dihydroxybenzoic[a]		19.28	18.28	18.99	18.11	0.88
3-Hydroxy-4-methoxybenzoic[a]		19.33	17.60	19.23	17.52	1.71
3-Hydroxy-4-methoxyhippuric[a]		26.34	23.26	26.31	23.18	3.13
3-Hydroxy-4-methoxyphenylacetic		19.39	17.58	19.30	17.48	1.82
β-(3-Hydroxy-4-methoxyphenyl)propionic[a]		20.45	18.71	20.42	18.68	1.74
3-Hydroxy-4-methoxycinnamic[a]		22.57	20.54	22.55	20.59	1.96
β-(3-Hydroxy-4-methoxyphenyl) hydracrylic[a]		21.18	20.04	21.06	19.88	1.18
4-Hydroxy-3-methoxybenzoic[a]		19.11	17.53	19.05	17.49	1.56
4-Hydroxy-3-methoxyhippuric[a]		24.88	23.27	24.71	23.32	
4-Hydroxy-3-methoxyphenylacetic[a]		19.38	17.61	19.33	17.54	1.79
β-(4-Hydroxy-3-methoxyphenyl) propionic[a]		20.53	18.84	20.57	18.83	1.74
4-Hydroxy-3-methoxycinnamic[a]		22.76	20.78	22.75	20.78	1.97
4-Hydroxy-3-methoxymandelic[a]		20.20	18.92	20.06	18.74	1.32
β-(4-Hydroxy-3-methoxyphenyl)lactic[a]		21.45	20.27	21.36	20.23	1.13
4-Hydroxy-3-methoxyphenylpyruvic[a]		23.34	21.67	23.29	21.76	1.53
4-Hydroxy-3-ethoxybenzoic[a]		19.55	18.04	19.46	18.02	1.44
3,4-Dimethoxybenzoic		19.47	16.84	19.44	16.76	2.68
3,4-Dimethoxyphenylacetic[a]		19.61	16.87	19.56	16.78	2.78
4-Hydroxy-3,5-dimethoxybenzoic[a]		20.90	18.89	20.83	18.80	2.03
3-Indolylcarboxylic[a]		22.60	19.95	22.78	20.01	2.77
3-Indolylacetic[a]	(i)	21.82	18.65	22.44	18.74	3.70
	(ii)	21.71	19.34	22.00	19.41	2.59
β-(3-Indolyl)propionic[a]	(i)	23.15	19.71	23.55	19.86	3.69
	(ii)	22.76	20.53	23.07	20.81	2.26
β-(3-Indolyl)lactic[a]	(i)	23.92	21.07	24.17	21.18	2.99
	(ii)	23.34	21.68	23.55	21.79	1.76
3-Indolylpyruvic		26.33	24.30	26.41	24.34	2.07
N-Acetyltryptophan[a]		25.65	24.00	25.82	24.11	
		27.60		27.62		
3-(5-Hydroxyindolyl)acetic[a]		24.31	22.06	24.29	22.03	2.26

[a] The presence, or reported presence, of this compound in human urine is known to the authors. Other compounds such as phenylacetic, phenylacetylglutamic and *m*-hydroxycinnamic acids may be present in urine after hydrolysis.

Chromatographic conditions

Chromatograms were run on two Pye 104 series, Model 64 machines with flame-ionisation detectors maintained at 310° and a hydrogen flow rate of 50 ml/min. Argon

TABLE II

METHYLENE UNIT VALUES OF AMIDES AND INDOLES SILYLATED WITH HMDS-TFA

Acid		*Column*		
		OV-17	*OV-1*	*ΔMU (OV-17– OV-1)*
Hippuric		21.20	18.08	3.12
o-Hydroxyhippuric		23.11	20.52	2.59
m-Hydroxyhippuric		23.82	21.22	2.60
p-Methoxyhippuric		24.67	20.85	3.82
3-Hydroxy-4-methoxyhippuric		26.33	23.17	3.16
4-Hydroxy-3-methoxyhippuric		26.39	23.29	3.10
Phenaceturic		21.84	18.45	3.39
p-Methoxyphenaceturic		24.88	20.85	4.03
Phenylacetylglutamine		*ca.* 28.5	21.86	*ca.* 6.6
Phenylacetylglutamic		26.11	23.04	3.07
p-Methoxyphenylacetylglutamic		*ca.* 28.8	25.23	*ca.* 3.6
N-Acetylphenylalanine		20.72	17.84	2.88
N-Acetyltyrosine		24.97	21.24	3.73
N-Acetyl-*p*-methoxyphenylalanine		23.56	20.13	3.43
N-Acetyltryptophan		*ca.* 29.0	23.76	*ca.* 5.8
		27.68[a]		
3-Indolylcarboxylic	(i)	23.63	19.44	4.19
	(ii)	22.76[a]	20.05[a]	2.71
3-Indolylacetic		22.51	18.68	3.83
β-(3-Indolyl)propionic		23.64	19.83	3.81
β-(3-Indolyl)lactic		24.20	21.12	3.08
3-Indolylpyruvic	(i)	27.01	23.42	3.59
	(ii)	26.42[a]	24.30[a]	2.12
3-(5-Hydroxyindolyl)acetic		25.21	21.70	3.51

[a] This "difficult" peak was formed only in small amount.

at 50 ml/min was used as carrier gas. Injection heaters were used to give an initial temperature at the point of injection about 80° above that at which programming started. The nominal rates of temperature programming on the two machines differed by about 10%.

Coiled columns of 4 mm I.D. were packed with Diatoport S (80–100 mesh) supporting 10% liquid phase. The four variations of liquid phase, column length, temperature of commencement and rate of programming employed were as follows: OV-17, 5.5 m, 180°, 1°/min; OV-17, 1.5 m, 100°, 2°/min; OV-1, 4 m, 170°, 1°/min; OV-1, 1.5 m, 100°, 2°/min.

The simple terms OV-17 and OV-1 employed throughout the text refer only to the longer columns. References to the shorter columns are always qualified since they were used only for subsidiary experiments of a comparative nature.

Silylations with BSA

In preliminary experiments employing pure BSA silylation appeared sometimes to be very complex. Thus although *o*-hydroxyhippuric acid dissolved readily in the reagent no peak was observed when the mixture was chromatographed immediately on OV-17. In the course of time, however, no fewer than three peaks made their appearance, in two cases only temporarily. Such complications were largely eliminated

when silylation was carried out in BSA-pyr and this reagent was used for most subsequent experiments.

Compounds (usually 1–4 mg, the larger quantities being used when MUs were high) together with 0.5 mg of each hydrocarbon standard were dissolved in 0.5 ml BSA–pyr and the mixture allowed to stand at least overnight before chromatography. It was frequently possible to silylate and chromatograph several compounds simultaneously without ambiguity. Results are recorded in Table I.

Most members of two groups of compounds, indoles and amides, usually gave multiple peaks although secondary peaks were in many cases neither sufficiently large nor sufficiently consistent in formation to merit inclusion in Table I, particularly in the amide series. Thus, owing to limited supplies of material, an investigation into the unexpectedly large difference in MU values (OV-17) between the isomeric hydroxymethoxy hippuric acids (Table I) necessitated several repeated chromatograms of the original extracts, with and without addition of extra BSA. It was noticed that in some experiments each isomer gave a small secondary peak of similar MU value to that of the major peak from the other isomer. Similarly several examples were noted where amides and indoles present in mixtures gave results differing from those recorded in Table I. For example in various experiments 5-hydroxyindolylacetic acid gave rise to two peaks, N-acetyltryptophan to only one peak, and a third peak MU value 21.12 on OV-17 was observed from N-acetylphenylalanine.

Further observations indicated additional discrepancies between the behaviour of some amides and indoles when alone and when added to urine extracts. Thus whilst 5-hydroxyindolylacetic acid seemed reliably to form only one peak in urine, indolylacetic acid gave sometimes two peaks and at other times only the peak of MU value 19.41 (OV-1) or 22.00 (OV-17). Hippuric acid, invariably present in urine extracts, gave in addition to the normal peak MU 21.13 (OV-17) variable amounts of a "difficult" peak MU 19.88 (17.91 on OV-1) not observed with the pure compound.

This latter peak appeared to be formed more easily if extracts were dried with particular care before silylation. In one such extract the proportion of this peak was increased by the addition of extra BSA, and still further increased when TMCS was then added. When similar extracts were silylated using BSA alone or BSA–TMCS formation of the "difficult" peak appeared to be complete, or nearly so, since only a small peak (which may have been due to some other compound) remained at MU 21.13 (OV-17).

The effect of moisture was investigated in an extract silylated with BSA–pyr which showed a particularly high proportion of the "difficult" peak from hippuric acid and both peaks from added indolylacetic acid. After the initial chromatogram on OV-17 water was gradually added to the extract by breathing gently into the container which was then shaken and allowed to stand 5 min before re-chromatography. Repetition of this process caused the gradual diminution of the "difficult" peak from hippuric acid and its eventual complete replacement by the normal peak. By contrast, the procedure resulted initially in a large increase of the peak MU 22.00 from indolylacetic acid at the expense of the peak MU 22.44, but little further change occurred subsequently. Since that of lower MU must be considered the "difficult" peak in the case of indolylacetic acid, water may well have a catalytic effect on its formation (*e.g.* ref. 5).

Silylations with HMDS

Silylation of simpler aromatic acids may be effectively achieved with HMDS–

pyr. The reaction is slow but may be speeded up by addition of TMCS (*e.g.* ref. 2). Neither variation proved satisfactory when applied to indoles and amides, whether pure or added to urine extracts. Results obtained appeared to be a function of time. Indoles gave double peaks, the relative proportions of which varied with time. Peaks were formed slowly from amides, sometimes obviously in very poor yield. Products were unstable in the presence of TMCS (stability was not tested with the uncatalysed reaction). Experiments with the above reagents were abandoned mainly because the insolubility of many substances, and of urine extracts in HMDS and/or pyridine made it impossible to devise any sort of consistent technique when dealing with them. The formation of a precipitate of ammonium chloride when TMCS was used also proved inconvenient.

It seemed possible that a catalyst alternative to TMCS could be found which would be free from the latter objection and would promote the rapid formation of homogeneous solutions. In point of fact the first, and only, two substances tried proved highly efficacious: addition of trifluoro- or trichloroacetic acid to suspensions of even highly insoluble substances such as acetyltryptophan in HMDS–pyr (1:1) resulted in rapid solution within a few minutes. When MUs of indoles and amides were redetermined (Table II) after silylation with HMDS–TFA solution of all substances, with one exception, occurred almost instantaneously and samples were injected after 15 min reaction time. The exception was phenylacetylglutamine which dissolved slowly, probably because the sample assumed the consistency of a gum when in contact with the reagent: reaction was allowed to continue overnight and the mixture was centrifuged to remove undissolved material which was probably inorganic in nature (personal communication from Dr. J. W. T. SEAKINS).

Under the above conditions substances gave single peaks with the exception of three indoles (Table II) which yielded apprecia bleamounts of secondary "difficult" peaks. However slow formation of 'difficult' peaks occurred with other indoles when the solutions were allowed to stand.

Further experiments on silylation

When indolyllactic, indolylacetic or 5-hydroxyindolylacetic acids were silylated (HMDS–TFA; 15 min) in the presence of indolylcarboxylic acid the latter two compounds gave traces of "difficult" peaks after 15 min, presumably as the result of a catalytic reaction involving the "difficult" peak formed relatively easily from indolylcarboxylic acid. Ether extracts were prepared in duplicate from 5 ml samples of six normal urines. To one of each pair of extracts was added indolylacetic and 5-hydroxyindolylacetic acids (1 mg each). After silylation (HMDS–TFA; 15 min) chromatography (OV-17) demonstrated the formation of "difficult" peaks from both indoles but in no case was the area estimated to be more than about 2 % of that of the corresponding normal peak. Areas of "difficult" peaks were approximately doubled when repeat chromatograms were carried out after 2.5 h reaction time.

A synthetic mixture containing hydrocarbon standards together with *p*-hydroxyphenyl-acetic and -lactic acids, indolylacetic and 5-hydroxyindolylacetic acids, N-acetylphenylalanine and *o*-hydroxy-, *m*-hydroxy- and *p*-methoxyhippuric acids was silylated under various conditions and chromatographed on OV-17 after suitable periods of time. One aliquot was treated with HMDS–TFA for comparison with results obtained when a second sample was silylated with BSA–pyr (24 h). Another aliquot

TABLE III

SILYLATION OF A SYNTHETIC MIXTURE OF ACIDS UNDER VARIOUS CONDITIONS

Equal aliquots of a mixture containing arbitrary quantities of acids were treated with various reagents (0.5 ml) and chromatographed on OV-17 after the time intervals indicated below. The figure recorded for each peak is the ratio of its height to that of a tetracosane standard.

Acid		*MU*	*HMDS-TFA*						*BSA-pyr*	*HMDS-TFA (15 min) then BSA*		*BSA*		*BSA-TMCS*
			15 min	*2.5 h*	*5 h*	*24 h*	*48 h*	*120 h*	*24 h*	*24 h*	*72 h*	*2 h*	*24 h*	*2 h*
p-Hydroxyphenylacetic		17.59	1.96	1.94	1.96	1.94	1.94	1.96	2.00	1.92	1.91	2.08	2.12	2.14
PHPL		19.86	0.82	0.78	0.85	0.83	0.81	0.82	0.84	0.81	0.78	0.88	0.92	0.91
Indolylacetic	(i)	22.48	0.83	0.83	0.82	0.73	0.68	0.50	0.05	0.00	0.00	0.77	0.67	0.78
	(ii)	22.00	0.01	0.02	0.03	0.10	0.18	0.35	0.72	0.99	0.97	0.19	0.29	0.09
5-Hydroxyindolylacetic	(i)	25.21	1.00	0.96	0.95	0.85	0.84	0.57	0.08	0.00	0.00	0.76	0.64	0.78
	(ii)	24.29	0.01	0.02	0.03	0.12	0.20	0.41	0.84	1.35	1.28	0.27	0.33	0.15
o-Hydroxyhippuric		21.03	0.77	0.79	0.80	0.77	0.81	0.74	0.27	0.75	0.69	0.08	0.03	0.03
m-Hydroxyhippuric		23.80	0.42	0.42	0.42	0.39	0.44	0.39	0.41	0.33	0.22	0.22	0.07	0.04
p-Methoxyhippuric		24.63	0.46	0.48	0.49	0.45	0.51	0.44	0.60	0.36	0.15	0.45	0.40	0.47
N-Acetylphenylalanine	(i)	20.66	0.59	0.56	0.59	0.59	0.59	0.59	0.04	0.35	0.32	0.02	0.02	0.00
	(ii)	19.31	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	0.27	–[a]	–[a]	0.38[b]	0.47[b]	0.58[b]

[a]Traces of this peak could have been obscured by a small overlapping artifact peak.
[b]An additional peak MU 21.13 was probably derived from N-acetylphenylalanine.

was also treated with HMDS–TFA but after a portion had been withdrawn for chromatography (15 min) an equal volume of BSA was immediately added. Other aliquots were silylated with pure BSA and BSA–TMCS. Results are recorded in Table III; as an indication of quantitative relationships the ratios of peak heights to those of the tetracosane standard were employed.

This experiment demonstrated the satisfactory formation and stability under all conditions of the peaks from the two simple phenolic acids. Indoles formed "difficult" peaks slowly when silylated with HMDS–TFA or pure BSA; their formation with the latter reagent was promoted by the presence of pyridine but not by that of TMCS. The formation of the "difficult" peak (and of a third peak) from N-acetylphenylalanine occurred with BSA but not in a mixture previously silylated with HMDS–TFA although this procedure secured the exclusive formation of "difficult" peaks from indoles. Peaks from the remaining amides tended to be smaller and less stable in the presence of BSA than those formed in the presence of HMDS alone.

It should not be assumed that the various types of behaviour described above necessarily apply to urine extracts. For instance the high degree of stability of all derivatives formed by HMDS–TFA was not confirmed in the case of that from *p*-methoxyhippuric acid which, when formed in a urine extract, disappeared slowly over the course of a few days.

Applications to urine extracts

Acidified urines were extracted with ether as described previously[4]. Normally that volume of urine containing 10 mg creatinine was taken and the dried evaporated extract treated with HMDS-TFA (0.5 ml). When aliquots (10 µl) were chromatographed on OV-1 or OV-17 as described above the ionisation amplifier was set at 2×10^3.

For quantitative purposes internal standards were employed. Because of overlapping due to peaks naturally present, the choice of standard may vary from urine to urine but docosane appeared to be generally suitable for OV-17 though rather less suitable for OV-1 in some cases.

Using docosane (200 µg added to each extract) as standard the excretions of VMA (OV-17) and PHPL (OV-1 and OV-17) were determined in a series of urines collected at noon from 10 normal subjects. Quantities of each substance were determined from standard graphs relating quantity to the ratio of the peak heights of the substance to that of docosane. These graphs were linear over the range tested (0–100 µg substance/ml HMDS–TFA) though slight curves were obtained when columns were presumably not in perfect condition. Since peaks were obviously heterogeneous on OV-1, excretions of VMA were not determined using this column; for comparison with OV-17 the substance was estimated by periodate oxidation[6]. Results are included in Table V.

DISCUSSION

Silylation procedures

The extraction of aromatic acids from aqueous solution, conversion into suitable derivatives and subsequent gas chromatography presents few technical problems and a wide choice of potential methods is available. The principal difficulties lie in the quantitative, or at least reproducible, formation of derivatives and in the selection of

columns which will reliably isolate compounds of interest as single peaks. The ready conversion of phenolic, alcoholic and carboxylic acid groups to TMS derivatives with a variety of silylating reagents and the excellent chromatographic properties of the majority of such derivatives has ensured a permanent place for silylation in this field.

At an early stage in our own work we were very impressed by the simplicity attached to procedures involving the silylation of all appropriate groupings, but did indeed note minor differences in chromatograms from urine extracts silylated under differing conditions[4]. Furthermore we found it profitable to compare results recorded by two other groups working with similar compounds. Both DALGLIESH *et al.*[2] and KAROUM *et al.*[3] chromatographed TMS derivatives of aromatic acids on the liquid phase F60; derivatives were prepared with HMDS catalysed by TMCS, the former group using pyridine and the latter group dioxan, as solvent. MU values were in reasonable agreement, allowing for wide differences in chromatographic conditions, except in the cases of hippuric acid and indoles. It is now clear that such compounds have a tendency to form more than one derivative; the formation of some peaks may be justifiably described as "difficult" since it occurs more readily with the particularly powerful silylating reagent BSA (*cf.* also ref. 7).

There can be little doubt that silylation of NH groups is involved in the chemistry of both amides and indoles and provides a reasonable explanation of the formation of "difficult" peaks from the latter type of compound. However, having regard to the wide differences in behaviour between individual members which makes generalisation dangerous, we would hesitate to draw firm conclusions concerning the chemistry of silylation in the amide series. Thus we have no ready explanation for the formation of three peaks by N-acetylphenylalanine and phenylacetylglutamic acid and are doubtful if peaks of similar MU values formed from one compound by different procedures necessarily always represent the same derivative. For instance we noted that the hippuric acid peak MU 21.13 (OV-17) when formed in urine using BSA appeared to be of a distinctly less symmetrical shape than when formed under any other conditions. Further speculation would appear to be unprofitable in the absence of concrete evidence concerning chemical structure.

Our experiments with indoles and amides illustrate variously that both the formation and stability of TMS derivatives may be influenced by the silylating reagent, solvent and presence of other substances. It seems probable that water has catalytic as well as hydrolytic properties and may be of paramount importance in determining the course of silylations using BSA: our results using this reagent should be regarded as typical rather than invariable. Undoubtedly the behaviour of an indole or amide of interest in urine samples should be checked in detail as the occasion arises: optimum conditions of silylation may vary from compound to compound and from urine to urine. Present evidence suggests HMDS to be a reagent superior to BSA for routine purposes because of its lesser tendency to give "difficult" peaks. In our experience replacement of TMCS by TFA as catalyst greatly enhances the utility and convenience of the reagent. In view of the many unexplored possibilities, it may be that future work will reveal some combination of reagent, solvent and catalyst which will silylate amides and indoles without ambiguity, but chaims in this respect should not be based solely on the behaviour of pure compounds.

As an alternative approach to the gas chromatography of aromatic acids both DALGLIESH *et al.*[2] and KAROUM *et al.*[3] have studied silylation following conversion of

carboxyl groups to their methyl esters. This procedure has the disadvantage that it is not possible to methylate completely all carboxyl groups without simultaneously partially methylating exceptionally reactive phenolic groups. Double bonds are also susceptible to attack by diazomethane. Some compounds may therefore yield multiple peaks. We have not investigated the silylation of methyl esters from indoles and amides since it seems quite clear that problems due to the formation of "difficult" peaks are again present. Not only are discrepancies in recorded MU values for these compounds apparent but the former group of workers observed the formation of two peaks from indolylacrylic and, on storage, indolylpyruvic acids.

MU values of aromatic acids

The behaviour of a compound on chromatography under given conditions is most accurately described in terms of its MU value which may readily be determined experimentally when flame-ionisation detection is employed. MU values are most closely related to molecular weights when non-selective liquid phases are employed and for this reason OV-1 may be regarded as a standard with which to compare the behaviour of liquid phases possessing selective properties. A number of such phases are now available in the OV-series: OV-17, a phenylmethylsilicone, was selected for detailed study. Most aromatic acids of biological interest are derived from the amino acids

TABLE IV

CHANGES IN MU VALUES OF CARBOXYLIC ACIDS WITH EXPERIMENTAL CONDITIONS

ΔMU values were usually obtained from Table I but values for amides and indoles were from Table II. δMU values were obtained from data in Table I.

Nucleus	*OH groups*[a]	*OMe groups*	*Number of examples*	*ΔMU (mean) (OV-17 − OV-1)*	*δMU (mean)*	
					OV-17	*OV-1*
Compounds lacking an NH group						
Phenyl	0	0	4	1.72	0.15	0.10
Phenyl	1	0	14	1.31	−0.03	−0.04
Phenyl	2	0	13	0.89	−0.14	−0.10
Phenyl	3	0	1	0.69	−0.17	−0.15
Phenyl	0	1	4	2.25	0.08	0.06
Phenyl	1	1	11	1.79	−0.04	−0.03
Phenyl	2	1	4	1.29	−0.10	−0.05
Phenyl	0	2	2	2.73	−0.04	−0.09
Phenyl	1	2	1	2.03	−0.07	−0.09
Compounds containing one NH group						
Phenyl	All amides		13	*ca.* 3.3	–[b]	–[b]
Indolyl	All indoles		6	3.67[c]	0.26[d]	0.10[d]
Compounds containing two NH groups						
Phenylacetyl-glutamine				*ca.* 6.6	–	–
N-acetyltryptophan				*ca.* 5.8	–	–

[a] Including enolisable keto groups.
[b] Data obtained from Table I in a few unambiguous cases do not reveal any marked influence of the amide group on δMU values.
[c] Excluding data from "difficult" peaks listed in Table II.
[d] Including data from both normal and "difficult" peaks.

phenylalanine, tyrosine and tryptophan and thus contain an aromatic nucleus linked to a side-chain containing no more than three carbon atoms. The list of compounds studied in this work is therefore rather comprehensive: it includes almost all aromatic acids at present known to be constituents of human urine and contains data sufficient to enable the prediction of MU values for many compounds not actually studied.

When a substance of unknown identity is encountered the most practicable approach to a consideration of its possible structural features may lie in a study of the variation of MU values with differing experimental conditions. Differential MU values may be derived in a number of ways; for instance values obtained using a non-selective column may be subtracted from those using a selective column. The ΔMU values (OV-17 – OV-1) listed in Tables I and II are summarised in Table IV and are always positive. However it is apparent that they are increased in magnitude by the presence of methoxyl and, particularly, the NH group present in amides and indoles, and progressively decreased in magnitude by the successive introduction of hydroxyl groups. Interpretation of ΔMU values obtained using OV-17 and OV-1 may be aided by the approximately equal magnitudes but opposite signs of the effects due to hydroxyl and methoxyl groups; differentiation between the presence in a molecule of mutually cancelling hydroxyl and methoxyl groups and the absence of such groups may be aided by a consideration of the approximate molecular weight as revealed by the MU value on OV-1.

Although MU values provide a fair description of the behaviour of a compound on a particular liquid phase they are not absolutely constant even with a given concentration of liquid phase, but depend on such factors as length of column and rate of heating if temperature programming be employed. For the purposes of this paper MU values were determined on short (1.5 m) columns at a fairly high rate of programming, conditions very suitable for routine work, as well as on the longer columns selected as being of more potential use for quantitative work. Differential MUs (δMU values) were obtained by subtracting values using the shorter from those using the longer columns, and are recorded in Table IV. Such values appear to be dependent almost entirely upon the number of hydroxyl groups present in a molecule, each of which exerts a negative effect. Unfortunately δMU values as recorded here are of little practical use since in many cases they are only of the same order of magnitude as experimental errors. However it might be possible to obtain values of greater magnitude by further attention to experimental detail.

MU values are influenced not only by the number of substituent groups but also by their nature (*e.g.* phenolic or alcoholic hydroxyl) and position (*e.g.* *o*-, *m*- or *p*-hydroxyl) and by the presence of double bonds as in cinnamic and enolised pyruvic acids. Such effects seem largely to be eliminated when differential MUs are considered though minor influences may be discerned. For instance, cinnamic acids appear to have particularly high ΔMU (OV-17 – OV-1) values and a hydroxyl group in the α-side chain position, as in mandelic and hydracrylic acids, seems to exert a particularly potent negative effect on δMU values. Interestingly, compounds of the above types appear to be revealed when differential MUs obtained by changing from a methylester-TMS derivative to a fully silylated derivative are considered. Examination of the data recorded by DALGLIESH *et al.*[2] and by KAROUM *et al.*[3] indicates that such changes produce increases in MU values which are particularly small in the case of mandelic acids and particular large in the case of cinnamic (and also hippuric) acids.

Applications to urine analysis

Previous experience suggested that most aromatic acids may be adequately extracted from urine using ether[4]. A few further experiments did not indicate any substantial advantage to be gained normally through further purification of acids by bicarbonate extraction since non-acidic urinary constituents seem to be excreted generally in much smaller quantities, often in conjugated forms poorly extracted into ether. Silylated ether extracts chromatographed very well (Fig. 1) and the principal difficulties appeared to be those associated with the complex behaviour of some substances as described above. In appropriate pathological urines large peaks were often observed and experience indicated unambiguous identification of such peaks to be greatly facilitated by the use of at least two columns. In some cases such as those of PHPL in tyrosinosis phenyllactic acid in phenylketonuria and homogentisic acid in alkaptonuria compounds were excreted in such large quantity that quantitative determinations could be undertaken without regard to the presence of overlapping normal peaks.

Quantitative determination in less spectacular cases presents considerable difficulty due to the possible heterogeneity of peaks. Given sufficiently extensive data on known compounds many problems may be solved in advance: for instance, reference to Table I indicates the futility of attempting VMA determinations on OV-1. However urines clearly contain many constituents as yet unidentified and under any given set of conditions few peaks on a chromatogram can be expected to be homogeneous. For the most accurate results possible quantitative determinations clearly need supporting evidence from ancilliary techniques such as mass spectrometry. However many unsupported estimations may be acceptable in certain circumstances. This is particularly true in clinical biochemistry where the normal may need to be defined only with sufficient precision to enable its distinction from the abnormal. It is also true when results can be shown to be reasonably close to those obtained by an alternative analytical method.

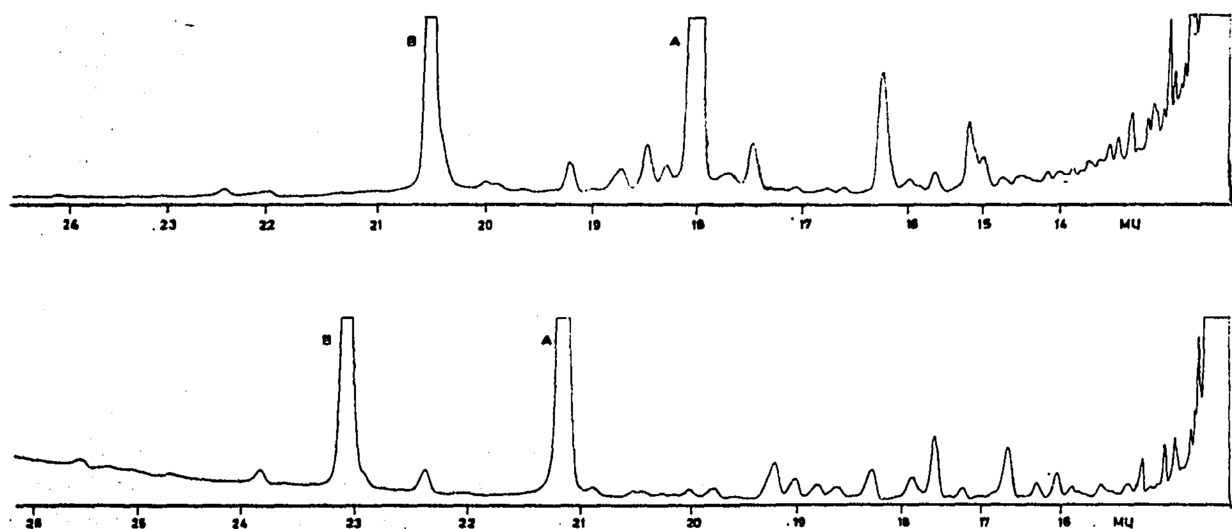

Fig. 1. Ether extract from a mixed normal urine, silylated with HMDS–TFA and chromatographed on OV-1 (above) or OV-17 (below). Base lines are marked in MU values to facilitate correlation of peaks with data in Tables I and II. Urines from normal adults invariably show the large peak A due to hippuric acid. The second large peak B, due to *o*-hydroxyhippuric acid derived from aspirin, is commonplace.

Quantitative aspects of the chromatography of silylated aromatic acids on OV-17 and OV-1 were examined in the cases of two of the most important urinary constituents of physiological interest. Both VMA and PHPL are commonly excreted in abnormal amounts in conditions of stress and in various diseases. Although VMA gave heterogeneous peaks on OV-1 comparison of results using OV-17 with those using a standard periodate-oxidation procedure indicated satisfactory agreement (Table V).

TABLE V

QUANTITATIVE DETERMINATION OF URINARY PHPL AND VMA

All values are expressed as μg/mg urinary creatinine.

Subject	*PHPL*		*VMA*	
	OV-17	*OV-1*	*OV-17*	*Periodate oxidation*
1	0.85	0.30	1.34	1.54
2	2.40	0.38	1.85	1.87
3	6.20	0.70	1.77	2.86
4	0.90	0.73	2.30	2.34
5	1.78	0.67	5.00	3.36
6	2.78	0.83	3.13	3.65
7	0.40	0.57	1.72	2.54
8	3.03	0.68	2.75	2.47
9	2.77	0.30	2.63	3.22
10	0.48	0.88	2.28	2.97

Determinations of PHPL on OV-1 proved preferable to those on OV-17 (Table V); however since the acid may increase enormously in pathological conditions either column should prove satisfactory for the detection of such conditions. The range of normal values for PHPL on OV-1 (0.30 – 0.88 μg/mg urinary creatinine) was considerably lower than that of 0.5 – 3.5 μg/mg creatinine reported recently when the same liquid phase was employed under isothermal conditions[8], despite the fact that in several urines the PHPL peak was obviously partially overlapped by another peak. This discrepancy may well reflect the limited accuracy possible in the determination of very small amounts by gas chromatography due to the difficulty in deciding the position of the base line from which measurements are to be made.

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