CHROM. 3915

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND ELUTION POSITION IN AN ANION EXCHANGE SYSTEM*

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SUMMARY

The elution positions of 114 reference compounds have been determined in the high-resolution anion exchange system being developed at the Oak Ridge National Laboratory for the analysis of body fluids. Compounds of the pyrimidine, amino acid, imidiazole, purine, pyridine, indole, quinaldic, and benzoic families of compounds and their derivatives elute in the approximate order of listing; however, extensive overlapping occurs. From consideration of the chemical family, the attached functional groups, the position of the attached functional groups, and the extent of conjugation, it is possible to make useful predictions of the elution positions of known compounds.

The regularities noted have been useful in predicting the elution position of known compounds and identifying compounds responsible for peaks. Predictions of peak positions are best made where related members of the same family have been tested.

INTRODUCTION

The identification of the compounds responsible for more than 150 peaks obtained by chromatography of human urine has been a significant problem in the application of the high-resolution, anion-exchange chromatograph in this laboratory[1,2]. Since the clinical evaluation of this analytical system at medical research establishments is imminent, the need for peak identification has become acute.

To date, specific compounds have been related definitely to 13 peaks, and tentatively to about 20 other peaks, found in urine chromatograms by the conventional techniques of co-chromatography plus mass spectrometry, nuclear magnetic resonance spectrometry, optical spectrometry, and chemical testing of separated fractions[3]. Characteristic of most of the identified compounds is the previous knowledge of their presence in large concentrations and their ultraviolet spectra. The identification of the remaining peaks will be difficult. The following factors contribute significantly to the difficulty: the presence of unknown compounds and conjugates, the large number of

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chemical families represented, the incomplete resolution of chromatographic peaks, the small quantities of the separated compounds (in many cases less than 1 μg), the low ultraviolet absorptivities of some of the compounds, and the admixture of gradient salt (exceeding the concentrations of the unknown compounds in some instances by a factor of $> 10^6$, by weight).

Additional promising techniques that are now being applied to this identification problem include: the collection of large quantities of the separated compounds with larger anion exchange columns and other chromatographic media[4]; the use of more-volatile gradient salts to facilitate their removal from collected fractions; adaptation of the gas chromatograph as an adjunct to the mass spectrometer; hydrolysis of the urine conjugates prior to analysis; polarography; improved separation of the observed peaks through alteration of the ion-exchange resin, the gradient system and other operating parameters; and a study of the relationship between elution position and chemical structure of eluted compounds.

This paper, which discusses the relationship between elution position and chemical structure, is limited to descriptions of the structural differences of 114 compounds and how these differences are related to retention on the column. For these compounds, the published pK values offer only a rough guide to the order of elution. In their pioneer work in this field, VOLKIN AND COHN[5] reported similar experience; the factors that modify the order of elution have been discussed by SOBER AND PETERSON[6], and COHN[7]. A theoretical study of the system is hampered because of the large number of chemical families involved, the limited number of compounds tested in some families, and the interdependent operating variables that must be considered. These variables include the nonlinear concentration gradient of the eluent, the nonlinear liquid flow rate, the change in column temperature, and the observed variation of the pH of the eluent.

EXPERIMENTAL DATA

The elution positions of 114 compounds were determined by chromatographing solutions of reference compounds, using the high-pressure, anion-exchange chromatographic system previously described[1,2]. These compounds were selected because: (1) they were considered to be probable constituents of urine, (2) they give useful ultraviolet spectra, and (3) they are commercially available in a relatively pure form. The elution positions of a small number of compounds, principally amino acids, were determined with radioactive tracers.

The elution positions of the reference compounds, shown superimposed upon a chromatogram of a urine sample in Fig. 1, illustrate the general distribution of peaks and compounds. To facilitate the discussion of chemical structure, the chemical families are assembled in Tables I–XI in approximate order of elution and in the order of elution within families.

DISCUSSION

The compounds that exhibited little or no retention on the anion exchange column include all those listed in Table I (basic and neutral compounds); one member

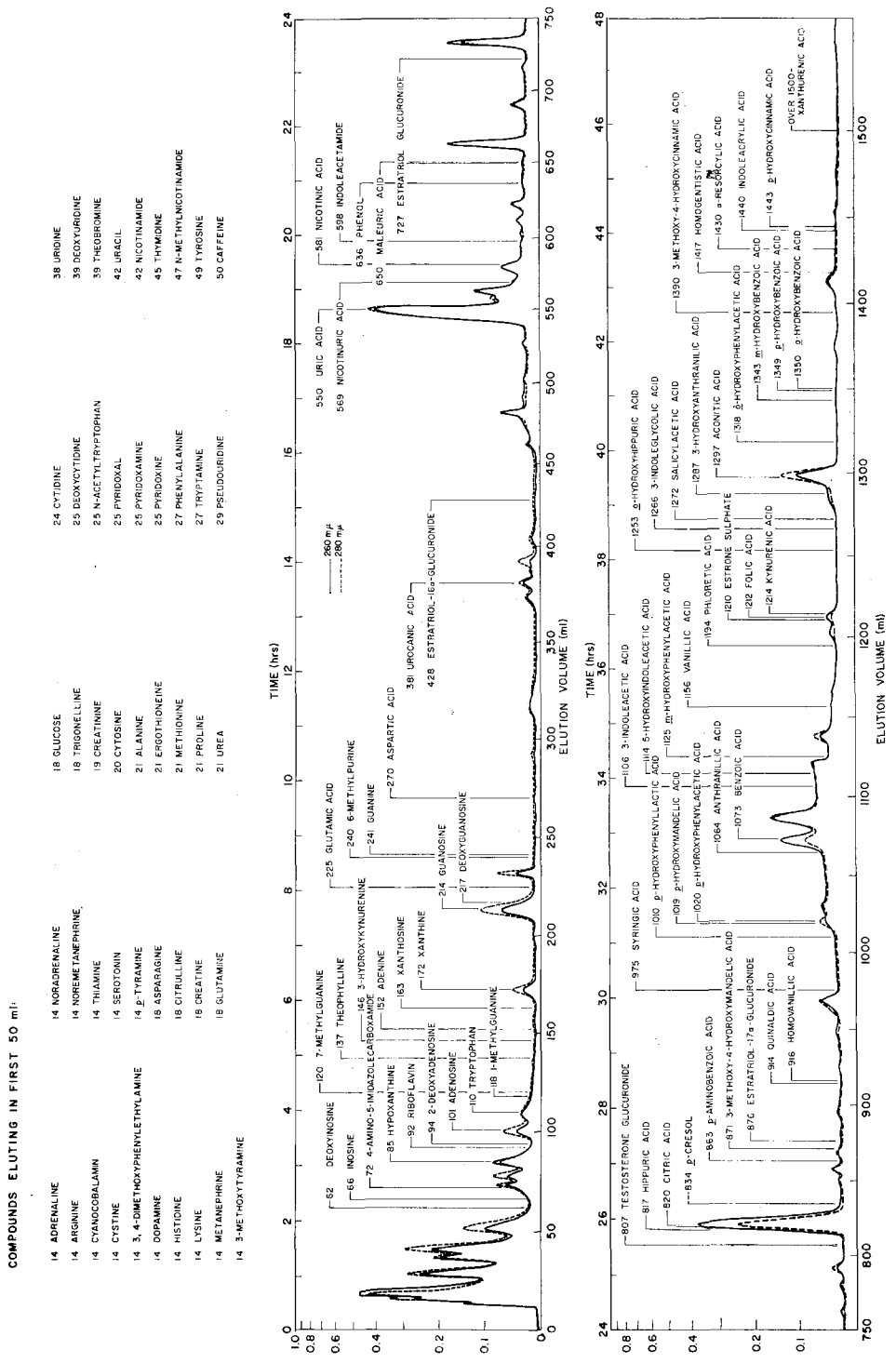


Fig. 1. A chromatogram of a urine sample showing position of known compounds, using urine reference sample No. 1.

each of the pyrimidine (Table II), imidazole (Table IV), and pyridine (Table VI) families; and a large number of the amino acids (Table III).

TABLE I

BASIC AND NEUTRAL COMPOUNDS

Compound	*Elution volume (ml)*
Adrenaline	14
Cyanocobalamin	14
3,4-Dimethylphenylethylamine	14
Dopamine	14
Insulin	14
3-Methoxytyramine	14
Metanephrine	14
Noradrenaline	14
Normetanephrine	14
p-Tyramine	14
Glucose	18
Urea	21

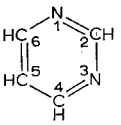
Basic and neutral compounds (Table I)

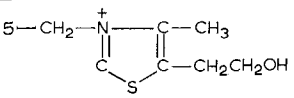
Aromatic bases and weakly ionized or neutral compounds were eluted at a single column volume, *i.e.* at 14 ml, and with no retention. In no case (except for the amino acids) did a compound having a carboxyl group or a carbonyl group with an α-hydrogen elute at 14 ml. However, many of the early-eluting compounds contained functional groups, such as hydroxyl, amide, imide, methoxy, etc., which apparently had no effect on their elution volumes. Urea (at 21 ml) and glucose (at 20 ml) were eluted later than might be expected. Thiamine (at 14 ml) and ergothioneine (at 21 ml), containing quaternary nitrogens, eluted near the breakthrough point, as expected.

TABLE II

PYRIMIDINE DERIVATIVES

Primary structure:



Compound	*Additions to pyrimidine structure*	*Elution volume (ml)*
Thiamine	2—CH_3, 4—NH_2, 	14
Cytosine	2=O, 4—NH_2	20
Cytidine	1—β-D-Ribofuranosyl, 2=O, 4—NH_2	24
Deoxycytidine	1—(2-Deoxy-β-D-ribofuranosyl), 2=O, 4—NH_2	25
Pseudouridine	1—H, 2=O, 3—H, 4=O, 5—β-D-ribofuranosyl	29
Uridine	1—β-D-Ribofuranosyl, 2=O, 3=H, 4=O	38
Deoxyuridine	1—(2-Deoxy-β-D-ribofuranosyl), 2=O, 3—H, 4—O	39
Uracil	1—H, 2=O, 3—H, 4=O	42
Thymidine	1—(2-Deoxy-β-D-ribofuranosyl), 2=O, 3—H, 4=O, 5—CH_3	45

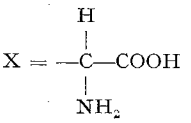
Pyrimidine derivatives (Table II)

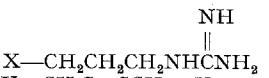
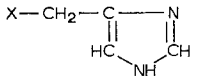
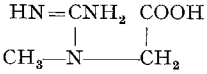
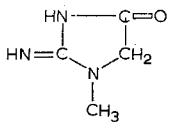
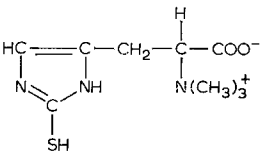
All members eluted early, with the relative position being strongly affected by the carbonyl group with an α-hydrogen. Three family members, each having one of these groups, eluted at 20–25 ml, whereas four other members, each having two of these groups, eluted at 29–45 ml. The inclusion of a carbohydrate structural element (and variations in that element) appears to shift the elution position by a few ml. As noted previously by COHN[8], the methyl group at position 5 in thymidine appears to favor later elution.

Riboflavin, while not a member of this family, resembles uridine and uracil structurally. Its large unsaturated cyclic structure appears to favor later elution (at 92 ml).

TABLE III

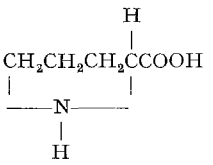
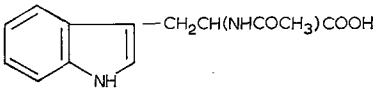
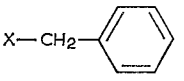
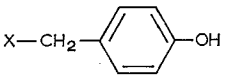
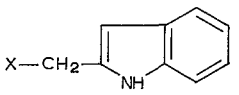
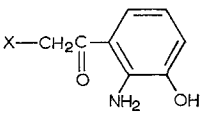
ORDER OF ELUTION OF AMINO ACIDS AND RELATED COMPOUNDS

Primary structure: X = 

Amino acid	*Structure*	*Elution volume (ml)*
Arginine		14
Cystine	$X—CH_2S—SCH_2—X$	14
Histidine		14
Lysine	$X—CH_2CH_2CH_2CH_2NH_2$	14
Asparagine	$X—CH_2CONH_2$	18
Citrulline	$X—CH_2CH_2CH_2NHCONH_2$	18
Creatine		18
Glutamine	$X—CH_2CH_2CONH_2$	18
Creatinine		19
Alanine	$X—CH_3$	21
Ergothioneine		21

(continued on p. 275)

TABLE III *(continued)*

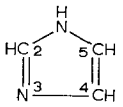
Amino acid	*Structure*	*Elution volume (ml)*
Methionine	$X—CH_2CH_2SCH_3$	21
Proline		21
N-Acetyltryptophan		25
Phenylalanine		27
Tyrosine		49
Tryptophan		110
3-Hydroxykynurenine		146
Glutamic acid	$X—CH_2CH_2COOH$	225
Aspartic acid	$X—CH_2COOH$	270

Amino acids (Table III)

The amino acids elute, as expected, in the following order: basic → aliphatic → aromatic → acidic. The aromaticity of phenylalanine (eluted at 27 ml), tyrosine (at 49 ml), and tryptophan (at 110 ml) favors longer retention times. The acetylation of the α-amino group of tryptophan reduces the elution volume from 110 to 25 ml. When the second carboxyl groups of glutamic acid (which is eluted at 225 ml) and aspartic acid (eluted at 270 ml) are blocked by amide groups to form glutamine and asparagine, respectively, the retention volume (18 ml) of each is approximately the retention volumes of the aliphatic monocarboxylic amino acids.

TABLE IV

IMIDAZOLE DERIVATIVES

Primary structure: 

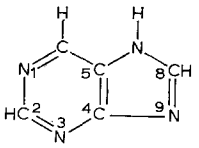
Compound	*Additions to imidazole structure*	*Elution volume (ml)*
Histidine	4-$CH_2CH(NH_2)COOH$	14
4-Amino-5-imidazole carboxamide	4-NH_2, 5-$CONH_2$	72
Urocanic acid	4-CH=CHCOOH	381

Imidazole derivatives (Table IV)

The imidazole structure appears to have less effect on retention on the column than other ring structures tested. Histidine elutes much earlier than the other aromatic amino acids, and urocanic acid elutes much earlier than comparable benzene derivatives (Table X) or the aliphatic unsaturated acids (Table VIII). The unsaturation in the side chain of urocanic acid, as in compounds of Table VIII and X, increases retention.

TABLE V

PURINE DERIVATIVES AND RELATED COMPOUNDS

Primary structure: 

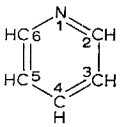
Compound	*Additions to purine structure*	*Elution volume (ml)*
Theobromine	1-H, 2 =O, 3-CH_3, 6 =O, 7-CH_3	39
Caffeine	1-CH_3, 2 =O, 3-CH_3, 6 =O, 7-CH_3	50
Deoxyinosine	1-H, 6 =O, 9-(2-deoxy-β-D-ribofuranosyl)	62
Inosine	6 =O, 9-β-*D*-ribofuranosyl	66
Hypoxanthine	3-H, 6 =O	75
Deoxyadenosine	6-NH_2, 9-(2-deoxy-β-D-ribofuranosyl)	94
Adenosine	6-NH_2, 9-β-D-ribofuranosyl	101
1-Methylguanine	1-CH_3, 2-NH_2, 3-H, 6 =O	118
7-Methylguanine	2-NH_2, 3-H, 6 = O, 7-CH_3	120
Theophylline	1-CH_3, 2 =O, 3-CH_3, 6 =O	137
Adenine	6-NH_2	152
Xanthosine	1-H, 2 =O, 3-H, 6 =O, 9-β-D-ribofuranosyl	163
Xanthine	1-H, 2 =O, 3-H, 6 =O	172
Guanosine	1-H, 2-NH_2, 6 =O, 9-β-D-ribofuranosyl	214
Deoxyguanosine	1-H, 2-NH_2, 6 =O, 9-(2-deoxy-β-D-ribofuranosyl)	217
6-Methylaminopurine	6-$NHCH_3$	240
Guanine	2-NH_2, 3-H, 6 =O	241
Uric acid	1-H, 2 =O, 3-H, 6 =O, 8 =O, 9-H	550

Purine derivatives (Table V)

As with the pyrimidine derivatives, the carbonyl group with an α-hydrogen strongly favors later elution. The most striking series is that of hypoxanthine (at 75 ml), xanthine (at 172 ml), and uric acid (at 550 ml), which have one, two, and three of these structural elements, respectively. When the α-hydrogen is replaced by α-methyl groups, as in theobromine, caffeine, and theophylline, these compounds elute earlier than the corresponding α-hydrogen analog (xanthine). An amine group in the 2 or 6 position favors later elution, as compared with the analogous carbonyl compounds; the methylamine groups appear more effective than the amine group (as in 6-methylaminopurine). As with the nucleosides of the pyrimidine family, these were separated from their base compounds only by a few ml.

TABLE VI

PYRIDINE DERIVATIVES

Primary structure: 

Compound	*Additions to pyridine structure*	*Elution volume (ml)*
Trigonelline	1-CH_3^+, 3-COO^-	15
Pyridoxamine	2-CH_3, 3-OH, 4-CH_2NH_2, 5-CH_2OH	25
Pyridoxal	2-CH_3, 3-OH, 4-CHO, 5-CH_2OH	25
Pyridoxine	2-CH_3, 3-OH, 4-CH_2OH, 5-CH_2OH	25
Nicotinamide	3-$CONH_2$	42
N-Methylnicotinamide	1-CH_3, 3-$CONH_2$	49
Nicotinuric acid	3-$CONHCH_2COOH$	569
Nicotinic acid	3-COOH	581

Pyridine derivatives (Table VI)

The basic and neutral members elute prior to 50 ml and the acid members at 550–600 ml. Trigonelline (which elutes at 15 ml) appears to be an exception; its carboxyl group may be neutralized by the quaternary nitrogen. The functional group in the third position increases retention in the order (of increasing effect): hydroxyl, carboxamide, and carboxyl. When the carboxyl group is conjugated, as in the case of glycine in nicotinuric acid, elution volume is decreased. The methyl group on the nitrogen atom improves the effect of the carboxamide in the third position, as in N-methylnicotinamide. From comparisons of the retentions of nicotinuric and nicotinic acids with those of hippuric and benzoic acids (Table X), it appears that the pyridine ring contributes to later elution less than the benzene ring does.

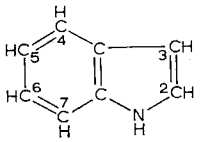
Indole derivatives (Table VII)

The elution volumes are, in most cases, approximately the same as those for analogous benzene derivatives (Table X). The acetamide, however, elutes later than might be expected.

TABLE VII

INDOLE DERIVATIVES

Primary structure:



Compound	*Additions to indole structure*	*Elution volume (ml)*
Serotonin	3-$CH_2CH_2NH_2$, 5-OH	14
N-Acetyltryptophan	3-$CH_2CH(NHCOCH_3)COOH$	25
Tryptamine	3-$CH_2CH_2NH_2$	25
Tryptophan	3-$CH_2CH(NH_2)COOH$	110
3-Indoleacetamide	3-CH_2CONH_2	598
3-Indoleacetic acid	3-CH_2COOH	1106
5-Hydroxyindoleacetic acid	3-CH_2COOH, 5-OH	1114
3-Indoleglycolic acid	3-CHOHCOOH	1266
Indoleacrylic acid	3-CH=CHCOOH	1440

Aliphatic acids (Table VIII)

The polar carboxyl group and unsaturation cause the late elution.

TABLE VIII

ALIPHATIC ACIDS OTHER THAN AMINO ACIDS

Compound	*Structure*	*Elution volume (ml)*
Maleuric acid	HOOC—CH=CH—CO—NH—$CONH_2$	650
Citric acid	HOOC—CH_2—COH(COOH)—CH_2—COOH	820
Aconitic acid	HOOC—CH=CH(COOH)CH_2—COOH	1297

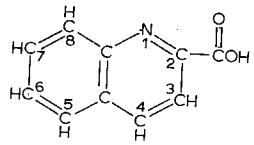
Quinaldic acid derivatives (Table IX)

The elution volumes are approximately the same as those for analogous benzene derivatives (Table X). The hydroxyl groups at positions 4 and 8 favor later elution.

TABLE IX

QUINALDIC ACID AND RELATED COMPOUNDS

Primary structure:

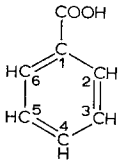


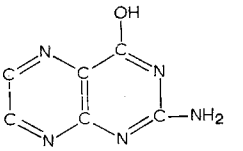
Compound	*Additions to quinaldic acid structure*	*Elution volume (ml)*
Quinaldic acid		914
Kynurenic acid	4-OH	1214
Xanthurenic acid	4-OH, 8-OH	>1500

TABLE X

BENZOIC ACID DERIVATIVES AND RELATED COMPOUNDS

Primary structure:



Compound (acid)	*Substituted for —COOH*	*Additions to benzoic acid structure*	*Elution volume (ml)*
Phenol	—OH		636
Hippuric	—$CONHCH_2COOH$		817
p-Cresol	—OH	4—CH_3	834
p-Aminobenzoic		4—OH	863
3-Methoxy-4-hydroxy-mandelic	—CHOHCOOH	3—OCH_3, 4—OH	871
Homovanillic	—CH_2COOH	3—OCH_3, 4—OH	916
Syringic		3—OCH_3, 4—OH, 5—OCH_3	975
p-Hydroxyphenyllactic	—$CH_2CHOHCOOH$	4—OH	1010
p-Hydroxymandelic	—CHOHCOOH	4—OH	1019
p-Hydroxyphenylacetic	—CH_2COOH	4—OH	1020
Anthranilic		2—NH_2	1064
Benzoic			1073
m-Hydroxyphenylacetic	—CH_2COOH	3—OH	1125
Vanillic		3—OCH_3, 4—OH	1156
Phloretic	—CH_2CH_2COOH	4—OH	1194
Folic	—CONHC(COOH) | HCH_2 | HC—COOH H		1212
o-Hydroxyhippuric	—$CONHCH_2COOH$	2—OH	1253
Salicylacetic		2—OCH_2COOH	1272
3-Hydroxyanthranilic		2—NH_2, 3—OH	1287
o-Hydroxyphenylacetic	—CH_2COOH	2—OH	1318
m-Hydroxybenzoic		3—OH	1343
p-Hydroxybenzoic		4—OH	1349
o-Hydroxybenzoic		2—OH	1350
3-Methoxy-4-hydroxy-cinnamic	—C=CHCOOH | H	3—OCH_3, 4—OH	1390
Homogentisic	—CH_2COOH	2—OH, 5—OH	1417
α-Resorcylic		3—OH, 5—OH	1430
p-Hydroxycinnamic	—C=CHCOOH | H	4—OH	1443

Benzene derivatives and related acidic compounds (Table X)

Basic and neutral derivatives that elute without retention on the column are discussed above (Table I). In an anion-exchange system, the thermodynamic stability of the anionic species (acid strength of the aromatic acids) (Table X) studied should be related to chemical structure and to retention on the anion-exchange column.

Since functional groups influence the reactivity or equilibria through inductive (electrostatic), resonance (conjugation), and steric effects[8], it should be possible to correlate elution volumes of aromatic acids with the functional groups present on the molecules. With some exceptions, such correlations appear in this family of compounds.

Benzoic acid, eluting at 1073 ml, may be considered as the base compound for the discussion that follows. The phenyl ring, through conjugation and induction, contributes to the stability of the benzoate ion; for example, benzoic acid was observed to elute later than the aliphatic acids (with the exception of aconitic acid). Compounds with functional groups that decrease the stability of the benzoate ion would be expected to elute earlier than benzoic acid; conversely, the compounds with functional groups that increase the stability should elute later. The compound *p*-aminobenzoic acid (eluted at 863 ml) illustrates the reduction of stability through resonance of the amino group in the para position, and the compound *o*-aminobenzoic acid (anthranilic acid), which is eluted at 1064 ml, shows increased stability through hydrogen bonding of the amino group in the ortho position. The inductive power of a meta hydroxyl group for stabilizing the anion is evident in the later elution of 3-hydroxyanthranilic acid (at 1278 ml).

On the basis of acid strength, the monohydroxy isomers of benzoic acid should elute in the sequence *p*-hydroxybenzoic acid, benzoic acid, *m*-hydroxybenzoic acid, and *o*-hydroxybenzoic acid. However, the sequence was found to be: benzoic acid (at 1073 ml), *m*-hydroxybenzoic acid (at 1343 ml), *p*-hydroxybenzoic acid (at 1349 ml), and *o*-hydroxybenzoic acid (at 1350 ml). The mono-hydroxy isomers of phenylacetic acid did elute in the predicted sequence of *p*-hydroxy (at 1020 ml), *m*-hydroxy (at 1125 ml), *o*-hydroxy (at 1318 ml). The high ionic strength of the eluting buffer (4–6 *M*) may affect the relative stabilities of the hydroxybenzoic acids more strongly than those of the hydroxyphenylacetic acids. The inductive effect of a *m*-hydroxy group on acid strength can be further illustrated with α-resorcylic acid (3,5-dihydroxybenzoic acid), which elutes at 1430 ml, as compared with *m*-hydroxybenzoic acid, which elutes at 1343 ml.

It has been shown above that the amine group in the ortho position of anthranilic acid, is capable of hydrogen bonding with the carboxyl group, thereby causing later elution. This effect was also noted with the hydroxyl groups of *o*-hydroxybenzoic acid and homogentistic acids. The hydroxyl group is more effective than the amino group in this respect; *i.e.*, the ortho hydroxyl compounds elute later than the ortho amino compounds.

The introduction of a methylene unit between the phenyl ring and the carboxyl group disrupts conjugation and results in acid weakening; this can be illustrated by the earlier elution of *p*-hydroxyphenylacetic (at 1020 ml) as compared with *p*-hydroxybenzoic acid (1349 ml) and by the earlier elution of homovanillic acid (at 916 ml) as compared with vanillic acid (at 1156 ml). An ethylene unit inserted between the phenyl and the carboxyl groups increases conjugation and results in acid strengthening, as illustrated by the late elution of 3-methoxy-4-hydroxycinnamic acid (at 1390 ml) and *p*-hydroxycinnamic acid (at 1443 ml), as compared with 3-methoxy-4-hydroxybenzoic (vanillic) acid (at 1156 ml) and *p*-hydroxybenzoic (1349 ml) acids.

Phenol and *p*-cresol, which are considered to be weak acids, are included as benzoic acid derivatives. As expected, they elute later than all basic and neutral

compounds but before most aromatic acids. *p*-Cresol, normally considered a weaker acid than phenol, unexpectedly elutes later than phenol.

TABLE XI

STEROIDS

Compound	*Elution volume (ml)*
Estratriol-16-α-glucuronide	428
Estratriol-3-glucuronide	727
Testosterone glucuronide	807
Estratriol-17-α-glucuronide	876
Estrone sulfate	1210

Steroid conjugates (Table XI)

The carboxyl group of the glucuronic acid moiety and the heterocyclic structure of the steroid moiety appear to be related to the later elutions of this family. The relatively late elution of the estrone sulfate probably results from the presence of the acid sulfate group.

CONCLUSION

The study of elution position as a function of chemical structure, rather than of pK values, was initiated for several reasons. First, the pK values are not available for many of the biological compounds of interest and, where available, may not be measured at the condition of interest. Second, as demonstrated by VOLKIN AND COHN[5] and SOBER AND PETERSON[6], the order of elution frequently does not follow the pK values, even in the case of simple systems. Third, the structure study complements other structural identification methods such as mass spectrometry and N.M.R. Finally, the method allows a small segment of the chromatogram to be considered so that linear correlation among family members becomes feasible. For example, the elution position of caffeine (at 50 ml) was predicted from the knowledge of related compounds in Table V.

A number of relationships that are consistent with accepted ideas of mechanism and structure[9] have been noted. In general, basic compounds elute first, followed by the neutral and then the acid compounds. Functional groups affect elution position most strongly in the case of acid compounds. Functional groups do not affect most basic compounds, which elute without retention; however, they do contribute to the separation within families of pyrimidines and purines and their nucleosides. Nucleosides differ only slightly from their bases in retention. It has been postulated that the retention of these compounds results from the affinity of the polystyrene matrix of the resin for these compounds[7]. COHN[7] considered purines to be more "benzenoid" than the pyrimidines and reported that alkylation increases affinity for the resin. The separations reported may be due to nonpolar binding, and the functional groups, depending on how they affect the nonpolar binding, may affect the elution position.

The amino acids elute earlier than most acid compounds. The basic and neutral

members elute with very little retention. Aromaticity, additional carboxyl groups, and additional hydroxyl groups increase the acid nature of the compounds and increase the retention volumes.

Indole and quinaldic derivatives and benzene rings make equivalent contributions to retention; imidazole and pyridine rings make less contribution. The basic and neutral aromatic compounds elute with little retention. The acid compounds are significantly retained; functional groups increase or decrease retention, depending on the nature of the group and its structural position on the molecule.

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