CHROM. 3993

AUTOMATIC LIQUID CHROMATOGRAPHY OF ORGANIC COMPOUNDS

I. MICROQUANTITATIVE ANALYSIS OF UREA TYPE COMPOUNDS BY AUTOMATIC LIQUID CHROMATOGRAPHY WITH A THERMAL DETECTION METHOD*

NOBORU NOMURA, DEN-ITSU SHIHO, KIICHIRO OHSUGA** AND MICHIKO YAMADA
Faculty of Pharmaceutical Sciences, University of Toyama, Gofuku, Toyama (Japan)

(First received November 13th, 1968; revised manuscript received January 24th, 1969)

SUMMARY

The separation of urea type compounds was studied by using thermal reaction energy detection automatic liquid chromatography. A strong polystyrene-based ion-exchange resin was used as adsorbent, activated in types of sulphonic acid, sodium sulphonate, and ammonium chloride.

The highest thermal detection sensitivity on liquid chromatography, ± 3/10000°, full scale was tested while changing the concentration of the eluent, namely 1.0, 0.1, and 0.01 *N* hydrochloric acid and water. It was illustrated that the sensitivity and reproducibility of the thermal reaction energy detection method were superior to those of colorimetry in quantitative microanalysis of urea type compounds.

INTRODUCTION

Urea type compounds are of great importance in the chemical and pharmaceutical field and also as products of protein metabolism. However, only a few workers have studied the separation and quantitative analysis of these compounds[1]. In automatic liquid chromatography, the detection method has to be applicable to all the components in the mixed sample. But unfortunately we have no report of a colorimetric method of detection common to all urea type compounds.

The authors propose to carry out the microquantitative analysis of urea type compounds by a universal thermal detection method in conjunction with automatic liquid chromatography. The thermal reaction energy detection method has been investigated by NAONO AND PŘCHAL[2] and TAMURA[3]. By using a thermistor as the

* Paper presented at the 10th Symposium on Liquid Chromatography, Kyoto, Japan, July 12th, 1966.

** Present address: Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Kyoto, Japan.

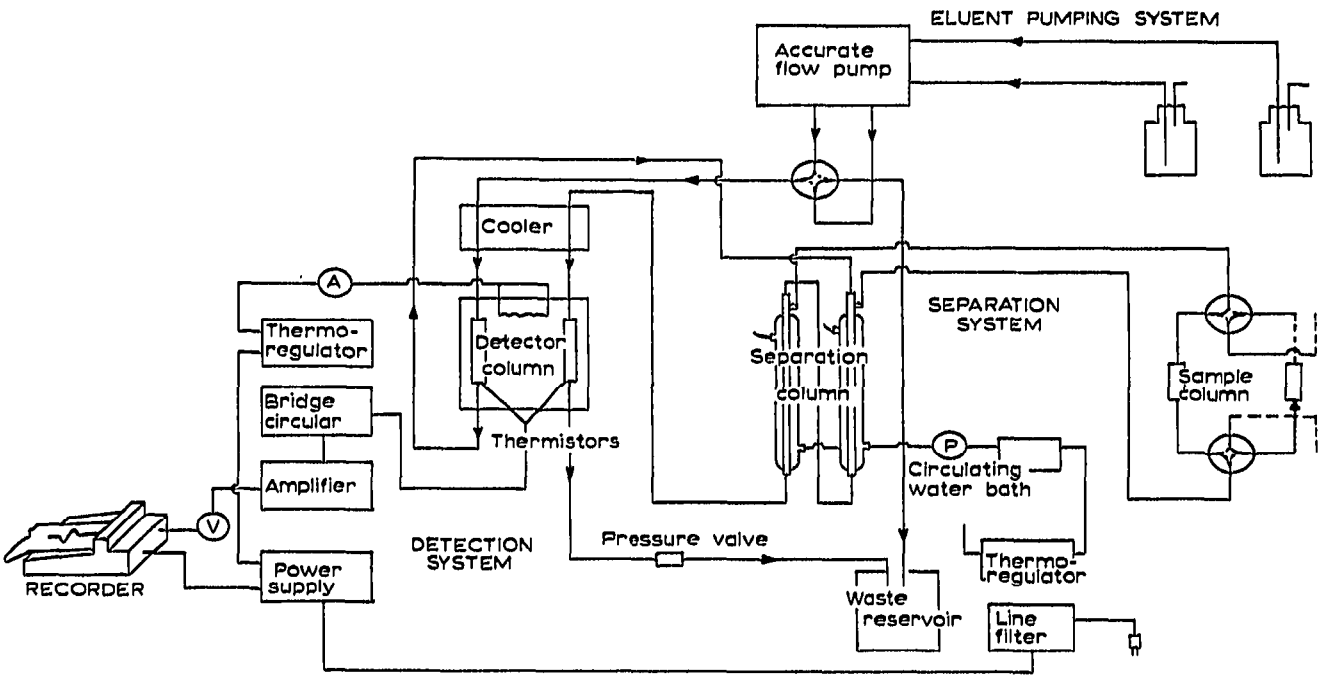


Fig. 1. Schematic diagram of thermal detection automatic recording liquid chromatograph (JLC-2A).

sensing element in the detection column, the variation in the heat of reaction between the sample and the packed material can be effectively measured. However, a means has to be devised to heighten the reaction energy and to eliminate the variation in the external temperature and noise from the electric circuit in order to increase the S/N ratio. When measuring over a period of hours at a time, the variation in the room temperature occasionally causes noise; thus the apparatus is set for the high sensitivity of $\pm$ 3/10 000° full scale.

The influence of the ion exchange resin and the eluent on the retention volume and the sensitivity of detection were examined. The authors obtained further information by measurements on the differential curve and from the peak heights and peak areas (as shown in Figs. 5 and 7a). As a result, the authors are confident that they can quantify urea type compounds by automatic liquid chromatography by use of the thermal reaction energy detection method. For microquantitative analysis of these compounds, the sensitivity and reproducibility of the thermal reaction energy detection method are superior to those of colorimetry.

THEORY

With regard to the thermal reaction energy detection method, ion exchange is a means by which the reaction energy can be intensified to the highest degree when the sample migrates between the stationary and the moving phase. In a neutral solution urea type compounds exist in a state of partial dissociation but after studying the adsorption mechanism of all these compounds under basic and acid elution conditions it was impossible to reach any conclusions regarding any increase in reaction energy. As the ion exchange process cannot be regarded as the only mecha-

nism, another adsorption mechanism must also play a role. The aromatic rings of the ion exchange resins of the polystyrene type have π-electrons, and the π-electron is capable of adsorbing many kinds of materials as molecules. Thus the authors presume that polystyrene type ion exchange resins tend to adsorb urea type compounds as molecules. After studying the effect that this type of exchange resin has on the adsorption of π-electrons, an attempt was made to find the conditions whereby the reaction energy between the resin and the urea type compounds is intensified to the highest degree.

Different ion exchange groups cause the variations in the swelling of the resin, and the variation in the swelling of the adsorbent affects the resolution. When these adsorbents are packed in a column, the reaction energy varies according to the adsorption and desorption processes, in addition sensitivity also varies according to column condition. However, the adsorption mechanism affects the sensitivity more than the column condition, though the variation of the column condition still has an effect on the retention volume. Therefore when the sample amount is constant, the smaller the retention volume, the narrower will be the base width of the peak, and the higher the peak height.

However, if the adsorption mechanism is changed, the above phenomenon does not apply. If the optimum adsorption condition is selected, even though the retention volume and the width are not so small, the peak height will increase. On the other hand, when the retention volume is large, the peak height should be increased by reducing the peak width by selecting the optimum measuring conditions.

In order to examine the reaction energy and the retention volume of the samples, three kinds of polystyrene type resin were used: a sulphonic acid type, a

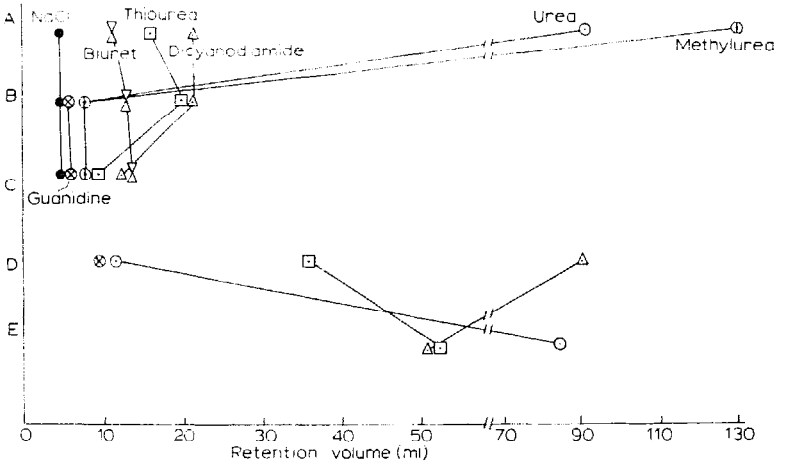


Fig. 2. Influence of the stationary phase on the retention volume of compounds related to urea in liquid chromatography. Stationary phases: (A) Amberlite CG-120, H^+, 325–400 mesh (resin particles regraded as described under EXPERIMENTAL); (B) Amberlite CG-400, Cl^-, 325–400 mesh (resin particles regraded as described under EXPERIMENTAL); (C) Amberlite CG-120, Na^+, 325–400 mesh (resin particles regraded as described under EXPERIMENTAL); (D) Amberlite IRA-400, OH^-, 48–80 mesh (TAKIMOTO *et al.*[1]); (E) Amberlite IR-120, H^+, 48–80 mesh (TAKIMOTO *et al.*[1]). Separation column, 0.8 × 15 cm (35°); detection column, 0.8 × 7 cm (30°); eluent, water; flow rate, 0.46 ml/min; sensitivity, $\frac{1}{1000}$ full scale. TAKIMOTO *et al.*[1] used a column 0.77 × 20 cm, water as eluent and flow rate 1 ml/min. The detection was accomplished by colorimetry.

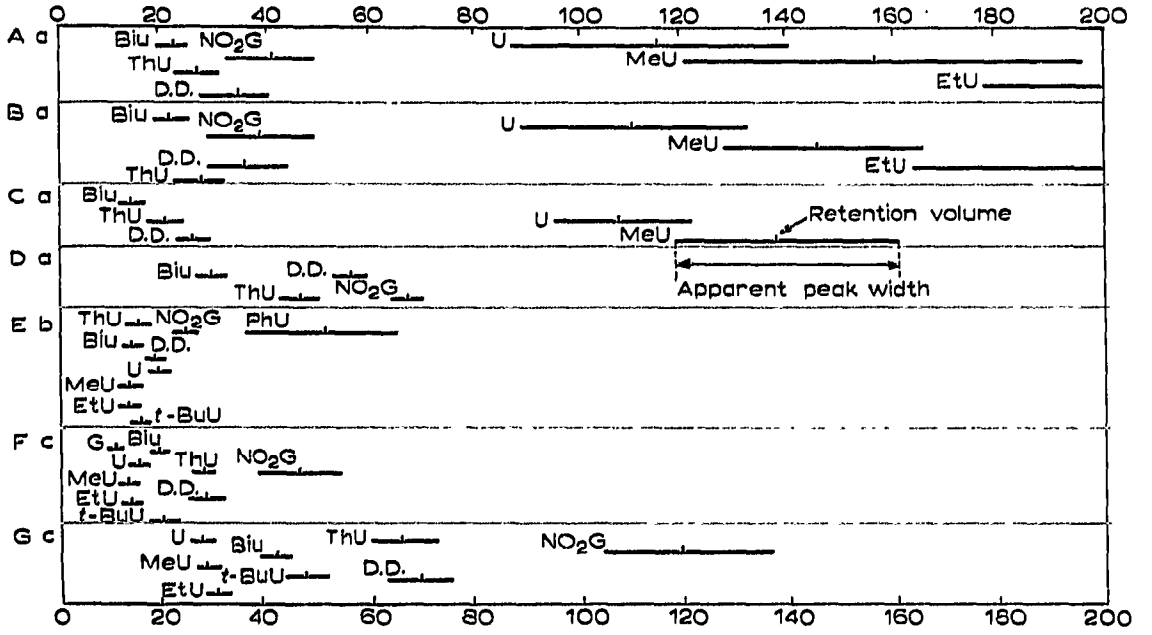


Fig. 3. Peak width and retention volumes of compounds related to urea in thermal detection liquid chromatography. Flow rate: 0.46 ml/min. Temperature: separation column, 35°; detection column, 30°. Separation columns: (A) Dowex 50 X8, H^+, 200–400 mesh, regraded, 0.8 × 15 cm, 0.01 *N* HCl; (B) Dowex 50 X8, H^+, 200-400 mesh, regraded, 0.8 × 15 cm, 0.1 *N* HCl; (C) Amberlite CG-120, H^+, 325–400 mesh, regraded, 0.8 × 15 cm, H_2O; (D) Aminex III X12, H^+, 400–600 mesh, 0.8 × 30 cm, H_2O; (E) Amberlite CG-120, Na^+, 325–400 mesh, 0.8 × 15 cm, H_2O; (F) Amberlite CG-400, Cl^-, 325–400 mesh, 0.8 × 15 cm, H_2O; (G) Amberlite CG-400, Cl^-, 325–400 mesh, 0.8 × 50 cm, H_2O. Detection columns: (a) Dowex 50 X8, H^+, 200–400 mesh, 0.8 × 7 cm; (b) Dowex 50 X8, Na^+, 200–400 mesh, 0.8 × 7 cm; (c) Dowex 1 X8, Cl^-, 200–400 mesh, 0.8 × 7 cm. Abbreviations: Biu = biuret; *t*-BuU = *tert.*-butylurea; D.D. = dicyanodiamide; EtU = ethylurea; G = guanidine carbonate; MeU = methylurea; NO_2G = nitroguanidine; PhU = phenylurea; ThU = thiourea; U = urea.

TABLE I

RETENTION VOLUMES OF COMPOUNDS RELATED TO UREA AS DETERMINED BY THERMAL DETECTION LIQUID CHROMATOGRAPHY

Flow rate: 0.46 ml/min. Separation column: 0.8 × 15 cm (35°). Detection column: 0.8 × 7 cm (30°). Sensitivity: ± 1/1000° full scale. Chart speed: 30 mm/h.

Compounds	*Amberlite CG-120, H^+*	*Dowex 50 X8, H^+*			*Dowex 1 X8, Cl^-*	
	H_2O	*0.01 N HCl*	*0.1 N HCl*	*1.0 N HCl*	H_2O	*0.01 N HCl*
Biuret	15.9	22.2	21.0	—	23.5	23.7
Thiourea	21.5	27.1	26.9	26.9	34.5	34.7
Dicyanodiamide	27.3	33.8	34.3	34.2	34.7	34.5
Nitroguanidine	—	40.7	37.5	—	—	—
Urea	106.7	113.1	108	58	18.4	18.2
Methylurea	136.6	154.1	145.1	—	18.9	18.4
Ethylurea	—	226	203	—	20.2	19.6
tert.-Butylurea	—	—	—	—	30.4	29.9

sodium sulphonate type, and a trialkylammonium chloride type. The experimental data of TAKIMOTO AND YAO, who used a trialkylammonium hydroxide type resin, are compared[1]. As the shape of the resin (spherical or crushed) and the linkage of the resin change the ion exchange capacity, these experiments were carried out under carefully controlled and constant conditions. The particles of commercially available resins were regraded.

Urea and methylurea showed very large retention volumes with respect to the sulphonic acid type resin, as depicted in Figs. 2 and 3. Conversely, these two compounds showed very small retention volumes on a sodium sulphonate type resin and a trialkylammonium chloride type resin. The variation in the eluent solution (water, 0.01 *N* and 0.1 *N* HCl) did not affect the retention volume when the sulphonic acid type was used as depicted in Table I. In the case where 1.0 *N* HCl was used as eluent, biuret, thiourea, and dicyanodiamide showed almost identical retention volumes, but the retention volume of urea was greatly decreased. Thus by increasing the hydrochloric acid concentration, the adsorption of urea was attained predominantly through the ion exchange mechanism; however, this was not true for biuret, thiourea, and dicyanodiamide. Accordingly, it is possible to increase the detection sensitivity of urea by increasing the hydrochloric acid concentration using the thermal reaction energy method. It is impossible, however, to increase similarly the sensitivity of biuret, thiourea, and dicyanodiamide. It is known that the pK_a of urea differs only slightly from those of biuret, thiourea, and dicyanodiamide, but the variation in pH in the eluent has a great influence on the contribution of the ion exchange mechanism to adsorption. This is very important not only in qualitative but also in quantitative analysis. Even a urea having a large retention volume increases its detection sensitivity in proportion to the decrease of hydrochloric acid concentration in the eluent

TABLE IIa

VARIATION IN PEAK HEIGHTS OF LIQUID CHROMATOGRAM OF COMPOUNDS RELATED TO UREA DUE TO A CHANGE IN STATIONARY PHASE AND ELUENT

Conditions, as in Table I.

Resin[a]	*Eluent*	*Peak height (mm) for 0.1 mg of sample*				
		Urea	*Thiourea*	*Dicyano-diamide*	*Biuret*	*Nitro-guanidine*
Amberlite CG-120, H^+ (325–400 mesh)	H_2O	18	20	38	33.5	—
Amberlite CG-120, Na^+ (325–400 mesh)	H_2O	40	59	80	72	—
Amberlite CG-400, Cl^- (325–400 mesh)	H_2O	25	80	80	57	50
Dowex 1 X8, Cl^- (200–400 mesh)	H_2O	20	60	55	36	—
Dowex 1 X8, Cl^- (200–400 mesh)	0.01 *N* HCl	17	56	54	35	—
Dowex 50 X8, H^+ (200–400 mesh)	0.01 *N* HCl	6.5	8.5	10.5	8.5	7.0
Dowex 50 X8, H^+ (200–400 mesh)	0.1 *N* HCl	4	6	6.5	6.5	6

[a] Resin particles regraded as described under EXPERIMENTAL.

with respect to the sulphonated type resin, as is summarized in Table IIa; the peak widths vary to a lesser degree, as is summarized in Table IIb.

Accordingly, this phenomenon is not explained by the simple mechanism of adsorption and so it is assumed that there is variation in the contribution of the adsorption mechanism. The bed permeability values of the adsorbent only vary by 2% if the hydrochloric acid concentration is changed from 1.0 *N* to 0 (water). Therefore the authors disregarded the permeability value of the adsorbent. The adsorption mechanism with regard to the ammonium type adsorbent (resin) is briefly summarized as follows:

TABLE IIb

VARIATION IN PEAK WIDTHS OF LIQUID CHROMATOGRAM OF COMPOUNDS RELATED TO UREA DUE TO A CHANGE IN STATIONARY PHASE AND ELUENT

Conditions, as in Table I.

Resin[a]	*Eluent*	*Peak width (ml)*				
		Urea	*Thiourea*	*Dicyano-diamide*	*Biuret*	*Nitro-guanidine*
Amberlite CG-120, H^+ (325–400 mesh)	H_2O	40.5	7.3	8.0	4.2	—
Amberlite CG-120, Na^+ (325–400 mesh)	H_2O	3.8	3.7	4.3	5.0	6.3
Amberlite CG-400, Cl^- (325–400 mesh)	H_2O	3.5	8.2	8.7	5.4	17.0
Dowex 1 X8, Cl^- (200–400 mesh)	H_2O	5.5	13.2	13.9	8.3	—
Dowex 1 X8, Cl^- (200–400 mesh)	0.01 *N* HCl	5.5	8.3	9.1	6.4	—
Dowex 50 X8, H^+ (200–400 mesh)	0.01 *N* HCl	54.7	11.0	14.4	8.9	19.3
Dowex 50 X8, H^+ (200–400 mesh)	0.1 *N* HCl	46.9	12.0	17.9	4.6	22.1

[a] Resin particles regraded as described under EXPERIMENTAL.

Guanidine and urea have very small retention volumes on the ammonium hydroxide type resin[1], while thiourea and dicyanodiamide have large retention volumes with regard to the same resin. Comparing the ammonium chloride type resin with the hydroxide type, the retention volumes of thiourea and dicyanodiamide decrease remarkably, but the retention volumes of the latter two compounds are still fairly large, compared with the retention volumes on the sodium salt type of sulphonated resin (Fig. 3).

The authors thought this comprised the essential difference between the two types of resin. However, in the case of an ammonium chloride type, each peak height obtained with 0.01 *N* HCl as the eluent was identical with the one obtained when water was used as the eluent. Furthermore the peak areas still tended to increase because the peak widths of thiourea and dicyanodiamide increased to a large degree and even that of biuret increased slightly.

EXPERIMENTAL

Sample

The samples used were recrystallized and their purity was examined by means of the melting point, organic microanalysis and infrared spectroscopy. The samples were weighed with an accuracy of ± 10 μg and the sample was dissolved in each eluent.

Equipment

In performing automatic recording liquid chromatography by the thermal reaction energy detection method, an elution tank constant flow rate pump, separation system, detection system and recorder were used (JLC-2A). This equipment was placed in a room having a constant temperature of 23 ± 1°. A diagram of the equipment is depicted in Fig. 1. The connections of the entire eluent system were made of Teflon tube (1 mm diameter). The detection system consists of a reference column and a detection column, which are in a high sensitivity thermostat. The sample flows from the separation column to the detection column, passing through the tube in the thermostat. Using a thermistor as the sensing element in the detection column, the variations in the heat of reaction accompanying sample elution could be effectively measured.

The variation in the reaction energy was recorded with a differential curve of the sample distribution (as shown in Figs. 4, 5 and 6).

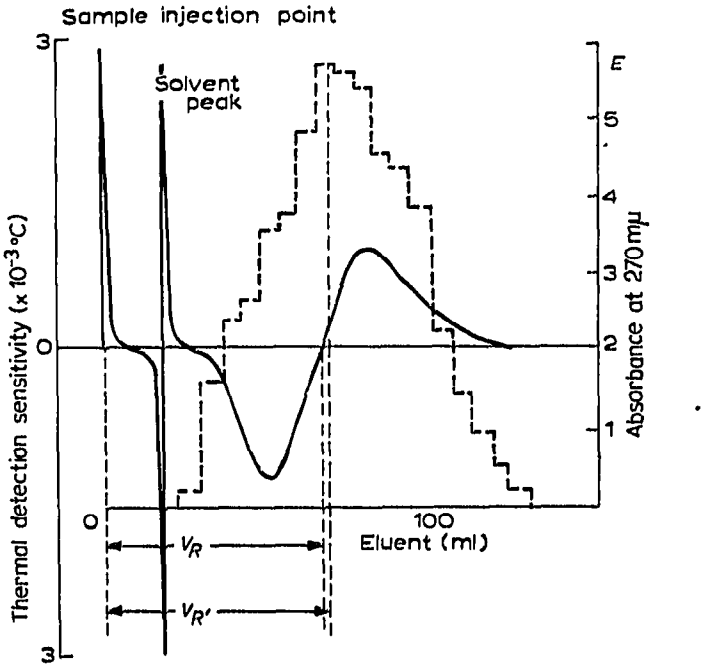
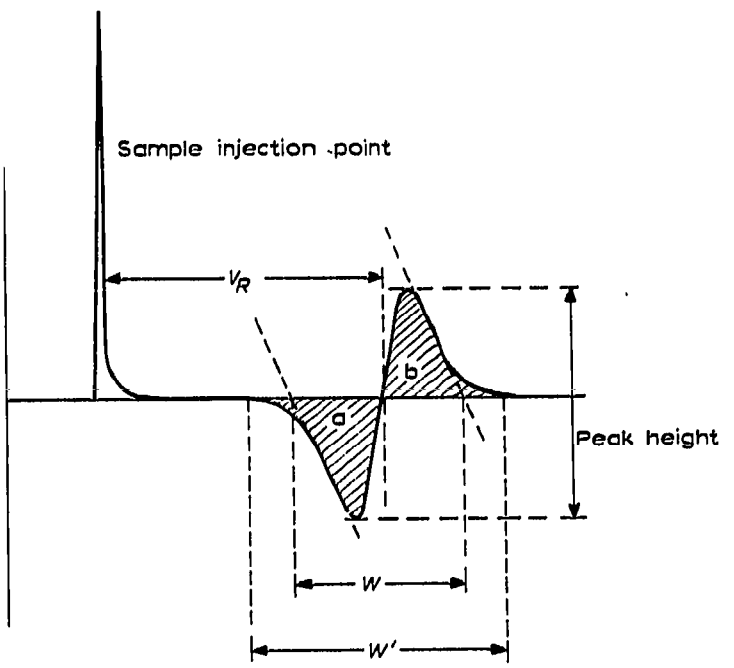


Fig. 4. Relationship between a thermal detection liquid chromatogram and the concentration distribution of cytosine. The absorbance at 270 mμ was measured using the fraction collector presented at the 9th Symposium of Liquid Chromatography, Tokyo, Japan, December 1, 1965. Sample: cytosine, 5 mg (4.5×10^{-5} *M*). Stationary phase: Dowex 50 X8, H^+. Eluent: 2 *N* HCl. Flow rate: 1.0 ml/min. Separation column: 0.8 × 15 cm (40°). Detection column: 0.8 × 7 cm (30°). Sensitivity: ± 3/1000° full scale. Chart speed: 30 mm/h. V_R = Retention volume of thermal detection chromatogram; $V_{R'}$ = retention volume of U.V. absorption detection chromatogram; $V_{R'} - V_R$ = tube volume between thermal detection column and fraction collector; V_R (corrected) = V_R (apparent) — tube volume between detector column and separation column.



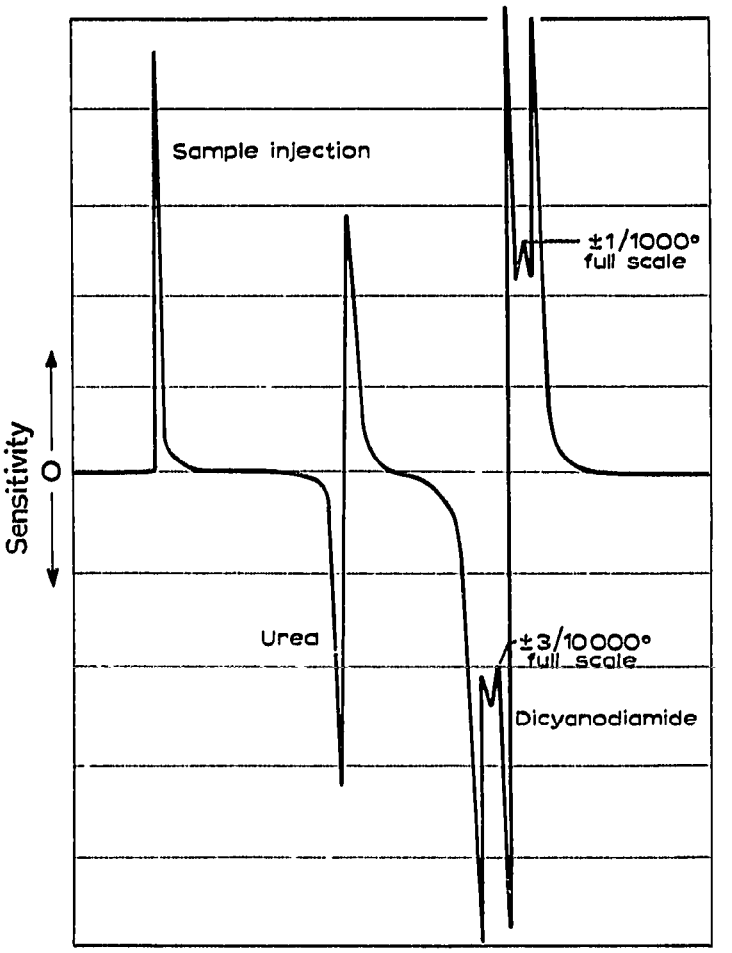


Fig. 5. Thermal detection chromatogram obtained as a differential curve. Peak area = total area of differential curve (a + b); V_R = apparent retention volume; W = corrected peak width; W' = apparent peak width.

Fig. 6. Automatic recording thermal detection liquid chromatogram of urea and dicyanodiamide. Stationary phase: Dowex 1 X8, Cl^-. Eluent: 0.01 N HCl. Flow rate: 0.46 ml/min. Separation column: 0.8 × 15 cm (35°). Detection column: 0.8 × 7 cm (30°). Sensitivity: ± 1/1000° and ± 3/10000° full scale for dicyanodiamide and urea, respectively. Chart speed: 30 mm/h. Samples: urea, 0.36 mg; dicyanodiamide, 1.00 mg.

Preparation of stationary phase

Regarding the particle size of resin, generally the smaller it is, the higher the separating efficiency will be. The mechanical rigidity of crushed resin is very weak compared with that of the spherical resin; further if the particle size is too small, the pressure required for a certain flow rate increases too much. Therefore, both the eluent and sample must be taken into account in determining the particle size of resin. Amberlite CG-120 type III, commercially available, was passed through a sieve to obtain a particle size of 325–400 mesh, and the particle size was further adjusted so as to be uniform by sedimentation as follows: The resin particles were allowed to swell in water and then a quantity of water, five or six times greater than the resin, was added to the resin. This mixture was then stirred well and allowed to settle for 5 min. The floating resin was taken off by decantation and the process repeated

part of the elution where the peptides containing relatively few ionic groups are eluted, a steep gradient would be flowing through the column; as the larger peptides (which are more sensitive to changes in salt concentration) begin to emerge from the column the gradient becomes progressively less steep. Experimental data supporting this conclusion have already been published[6]. It is important to note that peptides containing at least 16 ionic groups required a 0.1 *M* change in salt concentration from the time migration of these peptides was first detected until they moved as rapidly as the salt front through the column. This is significant because it implies that purification by "true" chromatography is possible as the peptides move down the column.

(D) Lysine peptides were found to have curved adsorption isotherms. Those peptides containing the greatest number of charges were found to be most sensitive toward changes in peptide concentration. Since many proteins and nucleic acids contain multiple ionic groups, and since curved isotherms cause very asymmetric elution profiles, the best resolution can be obtained when the smallest amounts of such polyelectrolytes are eluted from ion exchange columns, *i.e.*, the more symmetrical a peak the less tailing and possible overlapping there will be from one peak to another.

(E) Although, not investigated in this study, various theoretical considerations have shown that resolution is directly proportional to the square root of the column length, *i.e.*, increasing the column length fourfold brings about a twofold increase in resolution[20].

ACKNOWLEDGEMENTS

This investigation was supported by grants GPM-14, 672-R1, G-15298-02 of the National Institutes of General Medical Science of the National Institute of Health, and AI 00101 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

REFERENCES

1 H. A. SOBER AND E. A. PETERSON, *J. Am. Chem. Soc.*, 76 (1954) 1711.
2 E. A. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
3 F. E. BOMAN, in K. PEACH AND M. V. TRACY, (Editors), *Modern Methods of Plant Analysis*, Vol. 5, Springer, Berlin, 1963, p. 393.
4 G. SEMENZA, *Chimia (Aarau)*, 14 (1960) 325.
5 N. K. BOARDMAN AND S. M. PARTRIDGE, *Biochem. J.*, 59 (1955) 543.
6 J. W. STEWART AND M. A. STAHMANN, *J. Chromatog.*, 9 (1962) 233.
7 G. SEMENZA, *J. Chromatog.*, 18 (1965) 359.
8 M. A. SMITH, G. SEMENZA AND M. A. STAHMANN, *J. Chromatog.*, 18 (1965) 366.
9 M. A. SMITH, *Theoretical and Practical Aspects of the Chromatography of Polylysine on Carboxymethyl-Cellulose*, Ph. D. Thesis, University of Wisconsin, 1964.
10 E. A. PETERSON AND H. A. SOBER, in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in Enzymology*, Vol. 5, Academic Press, New York, 1962, p. 3.
11 M. A. SMITH AND M. A. STAHMANN, *J. Chromatog.*, 9 (1962) 528.
12 H. A. SOBER, F. J. GUTTER, M. M. WYCKOFF AND E. A. PETERSON, *J. Am. Chem. Soc.*, 78 (1956) 756.
13 E. HEFTMANN, in E. HEFTMANN (Editor), *Chromatography*, Reinhold, New York, 1961, p. 299.
14 D. H. HARRIS AND E. R. TOMPKINS, *J. Am. Chem. Soc.*, 69 (1947) 2800.
15 R. S. ALM, R. J. P. WILLIAMS AND A. TISELIUS, *Acta Chem. Scand.*, 6 (1952) 826.
16 J. W. STEWART AND M. A. STAHMANN, in M. A. STAHMANN (Editor), *Polyamino Acids, Polypeptides, and Proteins*, University of Wisconsin Press, Madison, 1962, p. 95.
17 N. K. BOARDMAN AND S. M. PARTRIDGE, *Biochem. J.*, 59 (1955) 543.

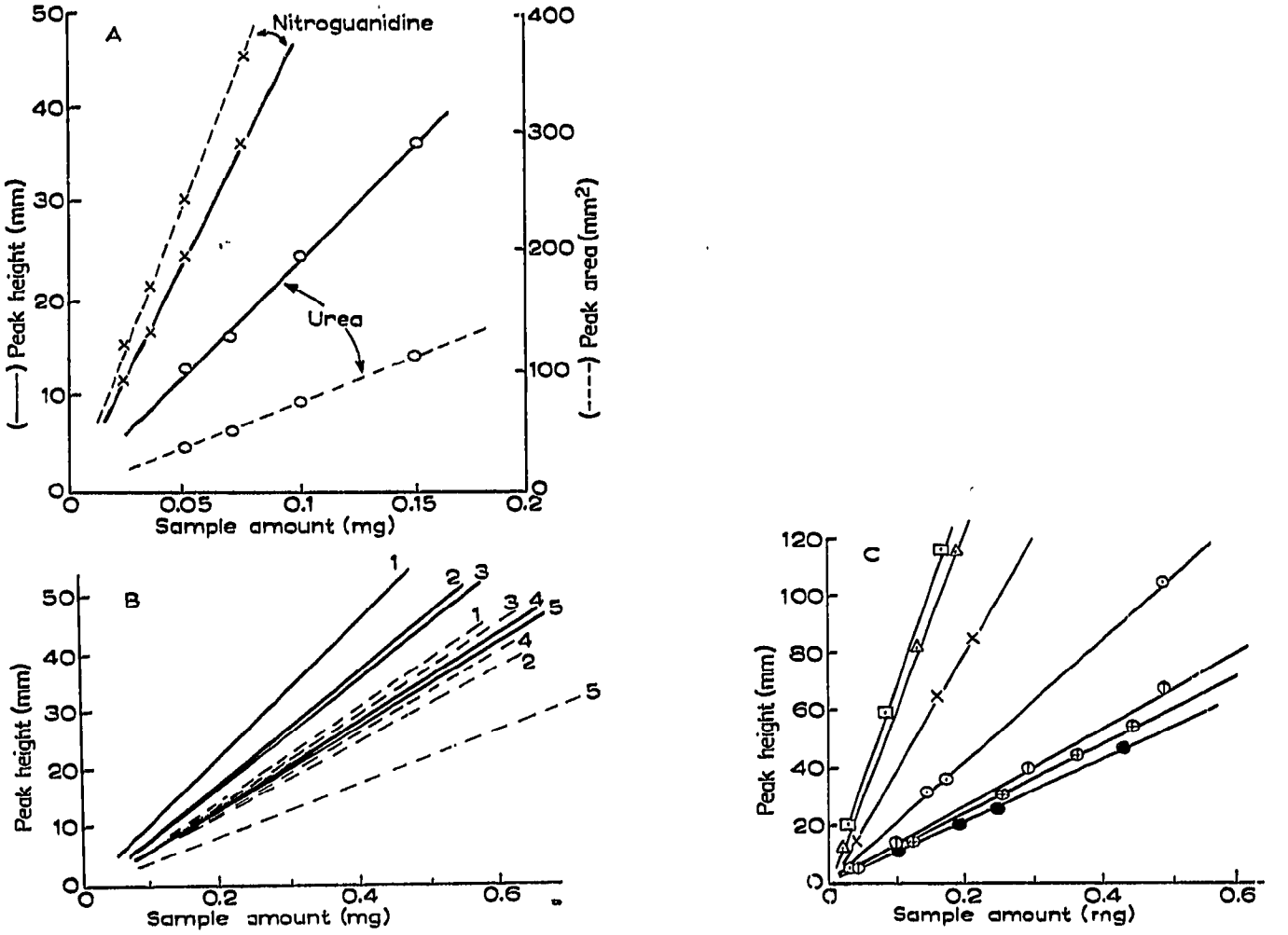


Fig. 7. (A) Calibration curves of urea and nitroguanidine obtained by measuring peak heights and peak areas. Separation column: Amberlite CG-400, Cl^-, 0.8 × 15 cm (35°). Detection column: Dowex 1 X8, Cl^-, 0.8 × 7 cm (30°). Eluent: water. Flow rate: 0.46 ml/min. Sensitivity: ± 1/1000° full scale. Chart speed: 60 mm/h. (B) Calibration curves of urea-related compounds in thermal detection chromatography. Separation column: 0.8 × 15 cm (35°). Detection column: 0.8 × 7 cm (30°). Stationary phase: Dowex 50 X8, H^+. Flow rate: 0.46 ml/min. Eluents: (———) 0.01 *N* HCl; (— — —) 0.1 *N* HCl. Sensitivity: ± 1/1000° full scale. Chart speed: 30 mm/h. 1 = Dicyanodiamide; 2 = thiourea; 3 = biuret; 4 = nitroguanidine; 5 = urea. (C) Calibration curves of urea-related compounds in thermal detection chromatography. Separation column: 0.8 × 15 cm (35°). Detection column: 0.8 × 7 cm (30°). Stationary phase: Dowex 1 X8, Cl^-. Flow rate: 0.46 ml/min. Eluent: water. Sensitivity: ± 1/1000° full scale. Chart speed: 30 mm/h. (⊙) urea; (⊕) methylurea; (●) *tert.*-butylurea; (⊡) thiourea; (⊕) ethylurea; (×) biuret; (△) dicyanodiamide.

injection pressure and the zero point of the differential curve. The volume of the tubes connecting the separation column and detection column was subtracted from the former volume. This difference was defined as the corrected retention volume (V_R) (as shown in Fig. 4).

The method of measuring the peak width is illustrated in Fig. 5. Because of the band-broadening effect between the separation column and the detection column, the measured peak width was larger than the true peak width. Figs. 7a, b and c are examples of the calibration curves.

CONCLUSION

A group of urea type compounds with pK_a values extending over a wide area of pH have been separated by thermal detection automatic liquid chromatography. 0.01, 0.1 and 1.0 *N* HCl and H_2O were used as eluents. Strong ion exchange resins with a polystyrene network were selected as the packing adsorbent for the detection column; these resins were of the ammonium chloride and sulphonated type, the latter being in the hydrogen and Na salt forms. The relationship between the chemical structure, retention volume, and peak area was studied, as was the relationship between the sensitivity, peak height and peak area.

From these results, the authors were able to show that it was possible to perform a microanalysis of these compounds. This method of analysis can be applied to other groups of compounds. It can also be employed for analyses of mixed samples. The actual detection sensitivity and the accuracy of quantity were very satisfactory, as illustrated in Figs. 7a, b, and c and Table III. A study of the conditions of measurement indicated that automatic recording liquid chromatography together with the reaction energy detection method is potentially a very effective method for separating and quantifying urea type compounds. Formerly the systematic separation and quantification of these compounds was very difficult.

TABLE III

REPRODUCIBILITY OF RETENTION VOLUME AND PEAK WIDTH OF A THERMAL DETECTION CHROMATOGRAM OF BIURET

Stationary phase: Dowex 50 X8, H^+. Eluent: 0.01 *N* HCl. Flow rate: 0.46 ml/min. Separation column: 0.8 × 15 cm (35°). Detection column: 0.8 × 7 cm (30°). Sensitivity; ± 1/1000° full scale. Chart speed: 30 mm/h.

Sample amount (mg)	V_R *(mm)*	W *(mm)*	*Peak height (mm)*
0.046	24.00	13.0	4.0
0.092	24.25	13.0	7.8 (7.75)
0.184	24.25	13.0	16.3 (16.25)
0.276	24.50	13.5	24.0
0.368	24.00	11.5	32.0
0.460	23.75	13.0	41.0
	$\bar{x} = 24.125$	$\bar{x} = 12.83$	

ACKNOWLEDGEMENTS

The authors are obliged to Dr. TOYOHIKO NAONO (Japan Optics Laboratory Co., Ltd.) for his useful discussion and the remodelling of the instrument, and Dr. RYO KOKUBO (Nissan Chemical Industrial Co., Ltd.) for his kind donation of samples. The authors also wish to thank Mr. M. MORIKOSHI and H. TAKAMI for their infrared spectroscopic measurements and organic microanalysis.

REFERENCES

1 M. TAKIMOTO AND T. YAO, *Nippon Kogyo Kagaku Zasshi*, 63 (1960) 1938.
2 T. NAONO AND K. PŘCHAL, *Ber. Bunsenges. Physik. Chem.*, 69 (1965) 900.
3 Z. TAMURA, *Bunseki Kagaku*, 14 (1965) 998.