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ANALYSIS OF BODY FUNCTIONS USING A CLINICAL LIQUID CHROMATOGRAPH

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SUMMARY

A clinical liquid chromatograph, which consists of a completely automated liquid chromatograph combined with a microcomputer for diagnosis, and its application to body function analyses are described. The analytical rate for urinary ultraviolet-absorbing constituents using anion-exchange chromatography was 12 samples per day, and the reproducibilities for retention time and peak area were less than 3% and 4%, respectively. Diagnostic methods for kidney functions using the chromatograms are discussed.

INTRODUCTION

It has been reported that much useful information on body functions can be obtained by the chromatographic analysis of components in physiological fluids[1]. Scott[2] demonstrated that an anion-exchange chromatographic system for ultraviolet-absorbing constituents in human urine had potential for diagnostic purposes, and he and his co-workers[3] developed an automated instrument for urine analysis. Although the analysis time was reduced to 8 h by using sequential columns of microreticular and pellicular resins[4], it was prohibitive for routine clinical use. Seta *et al.*[5] proposed a high-speed liquid chromatographic system with a macroreticular anion-exchange resin, by which 100 ultraviolet-absorbing constituents in human urine could be separated within 2 h.

In order to explore the potentialities of such systems, we have investigated the chromatography of urinary ultraviolet-absorbing constituents in human urine and its application to analyses of body functions. Since Scott[2] developed a linear gradient elution system with acetate buffer solution (pH 4.4) for chromatographing urinary ultraviolet-absorbing constituents, it has been used even for high-speed analysis using a macroreticular resin[5]. As described in a previous paper[6], we found that acetate

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buffer could not be used with a stepwise elution system because of the significant ultraviolet absorption of the eluents at 254 nm, resulting in baseline drift during the stepwise elution.

We developed a high-performance liquid chromatographic system, using stepwise elution with ammonium chloride–acetonitrile eluents, by which the analysis time was reduced to 90 min. Detection at 220–280 nm was possible with the system, so that ultraviolet absorption of the eluents was minimized. However, the eluent system needs a high concentration of ammonium chloride (2 mol/l) in the final eluent, which resulted in corrosion of the stainless-steel tubing and the separation column used in the chromatograph. Therefore, our later efforts were directed towards searching for a new eluent system, and finally it was found that addition of ammonium dihydrogen phosphate was effective in reducing the chloride concentration[7].

We report in this paper an automated apparatus for body function analyses,

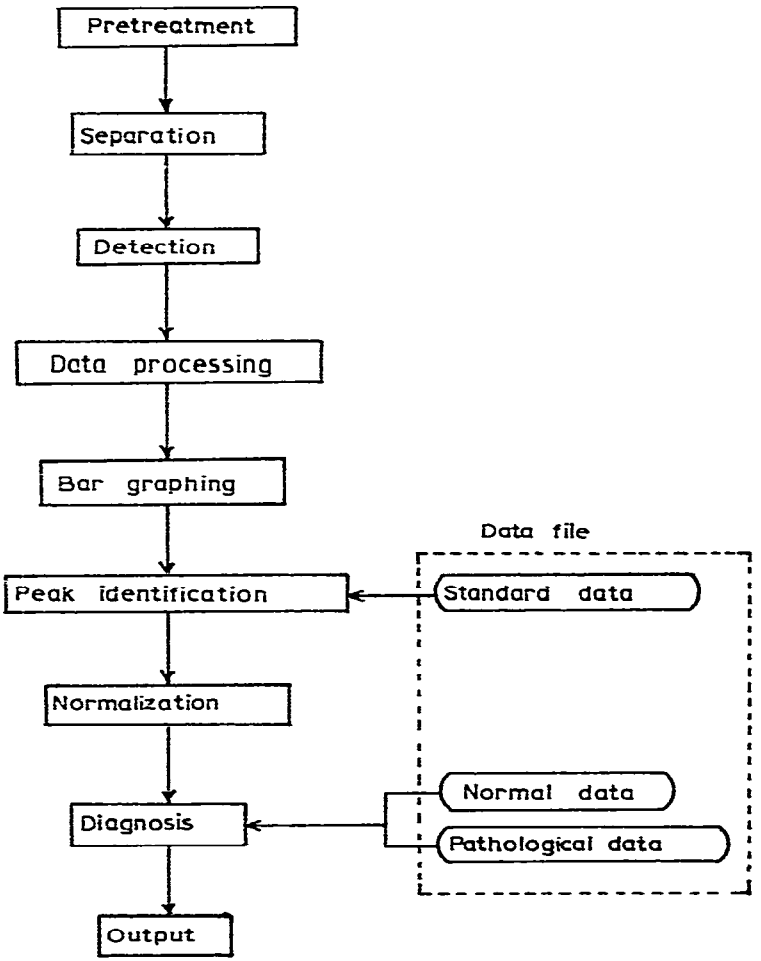

Fig. 1. General procedure for body function analysis.

which we call a clinical liquid chromatograph, based on a high-performance liquid chromatograph combined with a microcomputer for diagnosis.

PRINCIPLE

General procedure for body function analysis is illustrated in Fig. 1. After pretreatment to remove suspended or precipitated material, a urine sample is analysed with the liquid chromatograph, and ultraviolet-absorbing constituents are detected with a dual-wavelength ultraviolet monitor. The detected signals are digitized with a data processor, transferring the output signals to a microcomputer.

In order to effect peak matching, the peaks in the chromatogram are identified using the retention time and the absorbance ratio detected at two different wavelengths. Peak heights or peak areas of the identified peaks are normalized with respect to an appropriate peak to correct urine concentrations. Subsequently, diagnosis of the body functions is performed by several dignostic algorithms, for example, comparing total chromatographic profiles or comparing the ratio of the areas of two appropriate peaks. The diagnostic data are displayed on a screen or printed out.

EXPERIMENTAL

Apparatus

A schematic diagram of the clinical liquid chromatograph is shown in Fig. 2. An automated liquid chromatograph was constructed for the purpose described above, in which a dual-column system with two Model 835 pumps and a Model 635-0900 multiple wavelength ultraviolet monitor (Hitachi, Tokyo, Japan) were mounted. Seventy-two samples can be set simultaneously on the auto-sampler, which is cooled

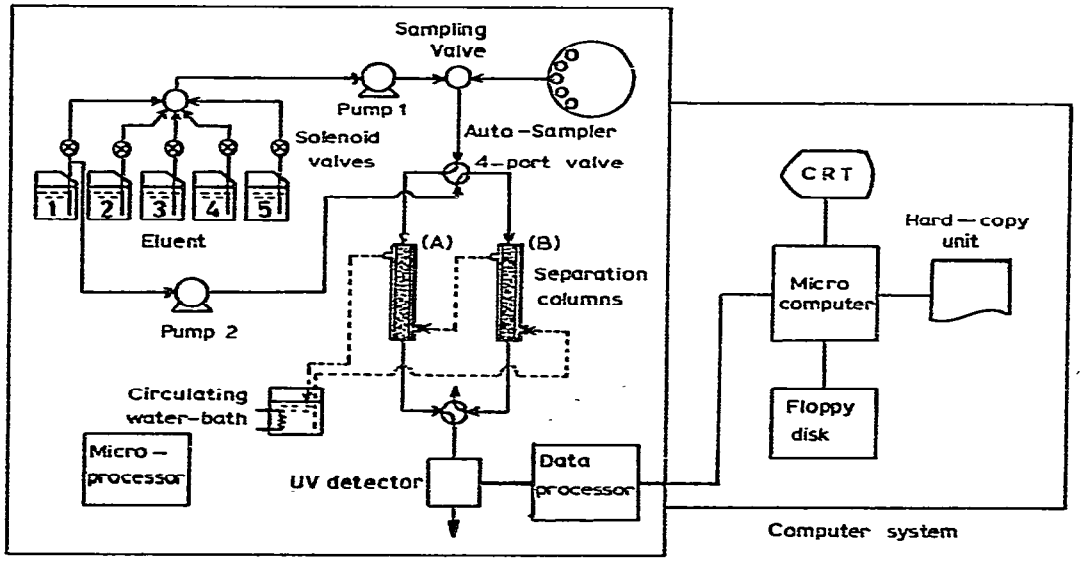

Fig. 2. Schematic diagram of clinical liquid chromatograph.

to 4°C with circulating water supplied by a Model BL-11 water cooling unit (Yamato, Tokyo, Japan). The eluent delivery system was based on stepwise elution, five or six consecutive eluents being supplied successively to the columns. A dual-column system was used to increase the analytical speed. Columns were switched with 4-port valves such that when one was used for the analysis, the other was used for re-equilibration with the first eluent. The column temperature was controlled by a constant-temperature circulating water-bath, and temperature programming was also possible. The detection wavelengths of the ultraviolet monitor can be chosen every 10 nm from 210 to 280 nm for each channel.

All of the analytical operation can be performed automatically by means of a microprocessor with respect to selection of eluent, column temperature, sample loading, column switching, etc.

The output signals of the data processor, which was mounted in the liquid chromatograph, were transferred to a microcomputer Model 9845 B/T with a graphic display and a hard-copy unit (Hewlett-Packard, Fort Collins, CO, U.S.A.). The chromatographic data were stored on a Hewlett-Packard Model 9845M floppy disk.

Anion-exchange chromatography

Stainless-steel columns of 250 × 4.6 mm I.D. packed with Hitachi Gel No. 3013-N macroreticular anion-exchange resin[6] were used for the separation of ultraviolet-absorbing constituents in urine. The resin was packed into the column by the slurry packing method. A new eluent system was developed for routine chromatographic analysis, consisting of ammonium chloride, ammonium dihydrogen phosphate and acetonitrile[7]. Under optimized conditions, the concentration of the final eluent was 0.3 mol/l ammonium chloride–0.05 mol/l ammonium dihydrogen phosphate–30 vol.-% acetonitrile (pH 4.8), and the eluents for the other steps, except for the first step (water), were prepared by dilution of the final eluent with water.

The column temperature was programed from 30 to 60°C synchronized with the eluent switching. The detection wavelengths of the ultraviolet monitor were selected at 230 and 250 nm.

Peak identification and diagnostic procedure

The chromatographic data digitized by the data processor were used for the diagnosis of body functions. First, peaks in the individual chromatograms were identified using the retention time and the absorbance ratio at 230 and 250 nm. The allowed variability of the identification parameters were fixed at ±1 min for the retention time and ±15% for the absorbance ratio. A commercially available chemical control urine was used to obtain a standard chromatogram.

Four kinds of diagnostic procedure were examined, the key parameters being (a) the chromatographic profile, (b) the normal range for an individual peak, (c) the peak height or peak area of an appropriate peak which is closely related to the traditional diagnostic parameter and (d) the relationship between two different peaks.

These diagnostic approaches were evaluated for the analysis of kidney functions.

Chemicals

Water and acetonitrile were purchased from Wako (Osaka, Japan) and were

purified for liquid chromatography. All other chemicals were of analytical-reagent grade. Reference urine was Q-PAK Chemical Control Urine (Hyland, Costa Mesa, CA, U.S.A.).

Urine samples were centrifuged with a Model 05RP-22 centrifuge (Hitachi, Tokyo, Japan) for 10 min at 3000 r.p.m., then the supernatant was pressure-filtered through a 0.1-μm pore size filter (Millipore, Bedford, MA, U.S.A.) before analysis.

RESULTS AND DISCUSSION

Anion-exchange chromatography of urine samples

A typical chromatogram of the reference urine obtained by stepwise elution with ammonium chloride–ammonium dihydrogen phosphate–acetonitrile is shown in Fig. 3. More than 80 peaks are separated within a 100 min under the conditions specified. The peak resolution and the analysis time are comparable to those obtained with the ammonium chloride–acetonitrile elution system[6]. As the eluent components have low ultraviolet absorption at wavelengths shorter than 250 nm, detection at 230 nm is possible without any baseline drift, as shown in Fig. 4.

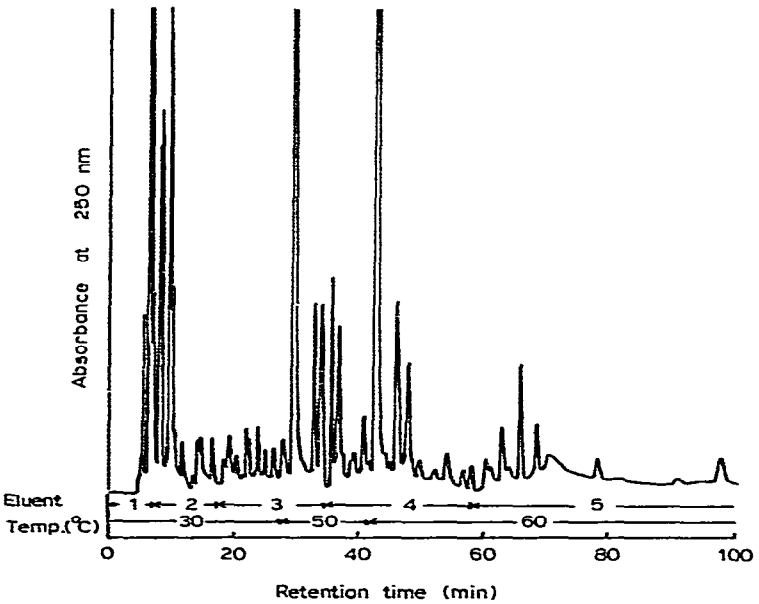

Fig. 3. Chromatogram of chemical control urine detected at 250 nm. Sample volume, 50 μl; column, Hitachi Gel 3013-N (250 × 4.6 mm I.D.). Eluent: (1) water; (2) 1/6 × (5); (3) 1/3 × (5); (4) 2/3 × (5); (5) 0.3 mol/l NH_4Cl–0.05 mol/l $NH_4H_2PO_4$–30% (v/v) acetonitrile (pH 4.8). Flow-rate, 0.8 ml/min; detector sensitivity, 0.32 full-scale.

We did not observe any corrosion problems with the eluents during 18 months' operation, because the chloride concentration was low, being one tenth of that in previous eluents.

The reproducibilities of the retention time, peak height and peak area are shown in Table I. A better reproducibility of the retention time was obtained for the

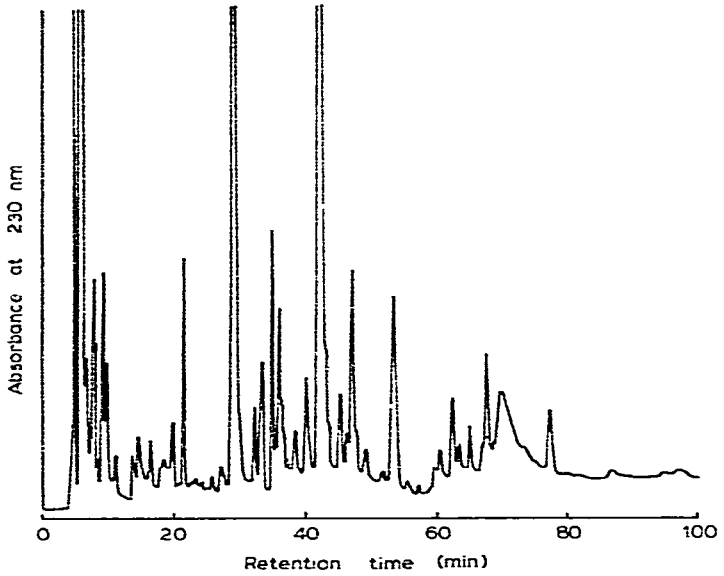

Fig. 4. Chromatogram of chemical control urine detected at 230 nm. Conditions as in Fig. 3.

peaks that were separated perfectly from neighbouring peaks (peaks 1 and 4) than for unseparated peaks (peaks 2 and 3). Instability of the baseline gave a lower reproducibility for peak height than peak area.

As would be expected, the long-term stability of the separation column depends on the number of samples loaded. It could be prolonged to more than 100 urine analyses by using a pre-column (10 × 4 mm I.D.) packed with the same packing material as the separation column.

The overall analytical rate with the apparatus was about twelve samples per day.

Peak identification

We examined the possibility of automatic peak identification with the microcomputer using the retention time and the absorbance ratio. The term "identification" is defined in this paper as discrimination of a peak in one chromatogram that is identical with a peak in a standard chromatogram.

TABLE I

REPRODUCIBILITY OF THE LIQUID CHROMATOGRAPH

No.	*Retention time (min)*	*Coefficient of variation (%) (n = 11)*		
		Retention time	*Peak height*	*Peak area*
1	5.32	0.82	0.78	2.27
2	18.40	1.92	7.27	3.23
3	33.80	2.70	8.38	3.32
4	74.20	0.89	8.12	2.80

Forty urine samples from normal subjects and a reference chemical control urine, used to obtain a standard chromatogram, were analyzed. Fifty peaks in the individual chromatograms were examined for identification with the criteria described above, and it was found that 25–35 of the 50 peaks could be identified. The reason why this number is lower than expected might be non-coincidence of the compounds involved in the reference urine and in the human urine, or the criteria used for the automatic identification, which did not allow for slight changes in the selectivity of the separation column.

Therefore, we checked the data for the chromatograms (retention times, peak heights, peak areas and absorbance ratios), then we numbered the peaks manually and input the peak numbers into the computer, these being used for diagnoses.

The peak identification process might be automated by increasing qualitative information such as the ultraviolet absorbance detected at 250–280 nm (ref. 3) or the intensity of fluorescence[8].

Diagnostic functions

In order to utilize the chromatographic data for diagnostic purposes, the chro-

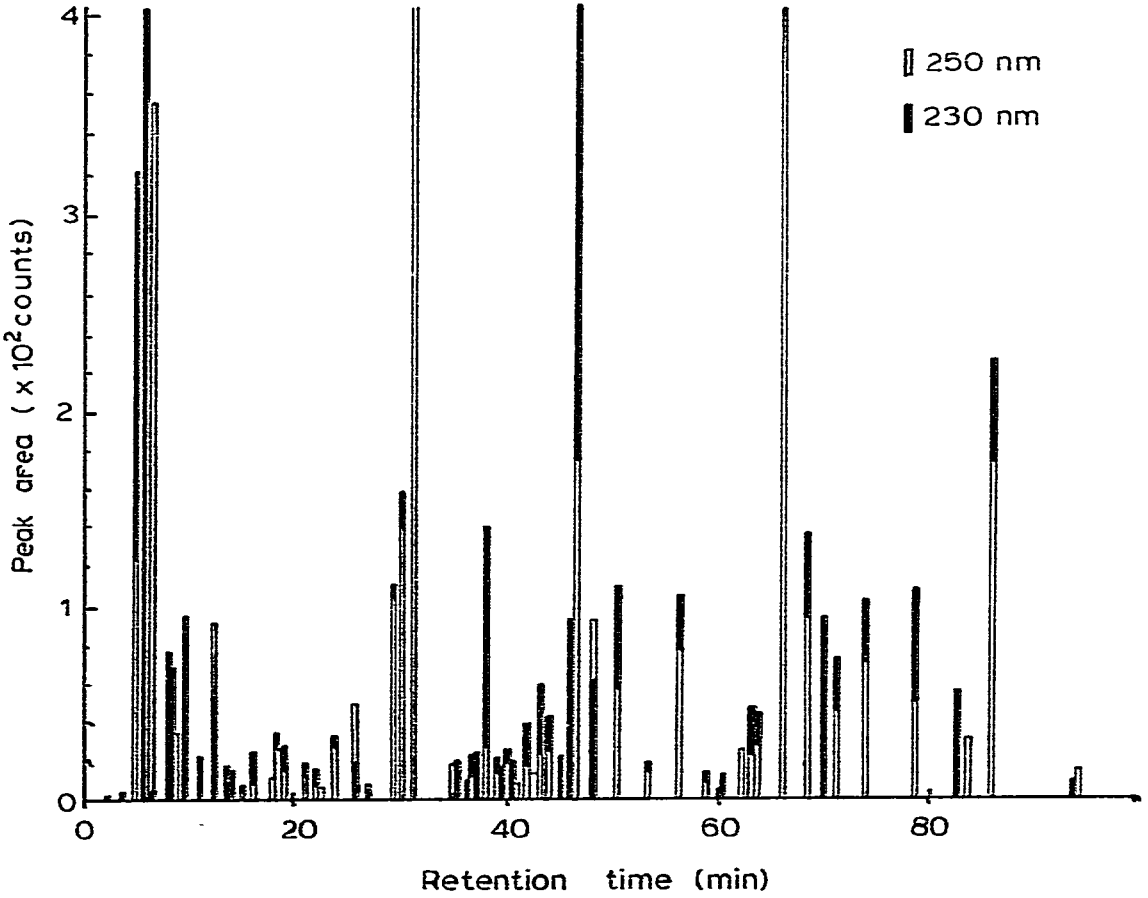

Fig. 5. Bar graph for urine of normal subjects reconstructed for body function analysis.

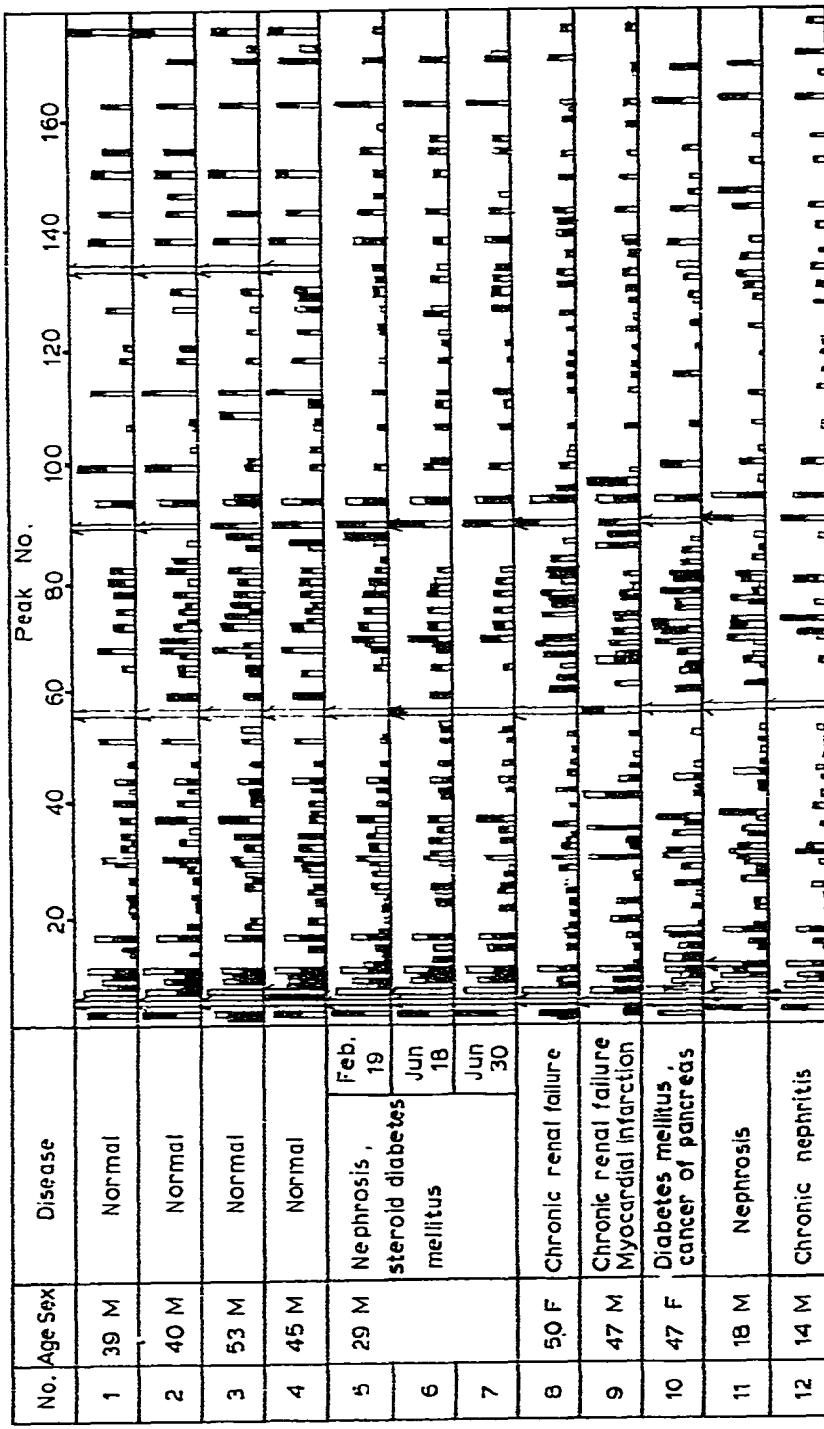

Fig. 6. Comparison of chromatographic profiles for various diseases.

matographic profiles[9,10] or concentrations of individual peaks calculated from the calibration graph or molar absorbance were used[11,12]. However, it is not so easy to make calibrations for many compounds using standard materials, because of a lack of pure standard compounds in some instances. Therefore, it is necessary to establish a diagnostic procedure using the chromatographic data without calibration.

We investigated such a diagnostic procedure, especially for the diagnosis of kidney functions, and found that the methods described below were promising.

All approaches tried in this work started from compiling a bar graph such as that shown in Fig. 5, which was composed automatically by the computer. Although the graph is simplified comparing with the original two chromatograms, sufficient data are included, namely retention time and peak area or peak height detected at 230 and 250 nm.

We analysed more than 300 urine samples, including 40 from normal subjects and 250 from patients with kidney disorders.

It is obvious that much useful information could be obtained by comparing the chromatographic profiles as shown in Fig. 6. For example, it was found that similar patterns were observed for normal subjects, samples 1–4; on the other hand, differences between normal and pathological patterns were observed for the region between peaks 100 and 180. We analysed urines from patients with nephrosis during five months (samples 5–7) and very similar chromatographic patterns were obtained, except for a slight difference in the region of peaks 110–150, which suggests a variation in the pathological situation.

It is very useful to compare chromatographic profiles such as those shown in Fig. 6 for diagnoses of body functions as described above. However, it is difficult to automate the process, because we cannot establish a normal pattern. Therefore, we tried to establish the normal range for individual peaks, instead of a normal pattern.

Urine samples from 40 normal subjects collected after dieting for 1 day were chromatographed, then the normal range was decided as ± 2 standard deviations of the mean value from the distribution graph of the peak area for each peak. In this instance, peak identification was performed automatically using a reference chemical control urine as a standard chromatogram, as described above.

Typical diagnostic graphs for a normal subject and a patient with chronic nephritis are shown in Figs. 7 and 8, respectively. Whereas seventeen peaks were identified and compared with the normal range in Fig. 7, only six peaks were identified in the patient's urine. In the pathological data, peaks which are out of the normal range are indicated by AAA (greatest), AA and A (smallest) according to the degree of shift from the normal range. It was again considered that the small numbers of peaks identified for the pathological urine might be due to the standard chromatogram.

It was felt that the automated diagnosis described above might provide a useful screening test if the probability of peak identification could be increased.

The urinary excretion rate of several compounds was examined by anion-exchange chromatography of ultraviolet-absorbing constituents in human urine[12,13]. Concerning to the diagnosis of kidney function, creatinine clearance is a most popular traditional diagnostic parameter. We tried to obtain information on creatinine clearance by using the liquid chromatographic system. In order to find the peak providing this information, correlations between the peak area of each peak and creatinine clearance were examined. Eventually, we found that peak 17 had the best

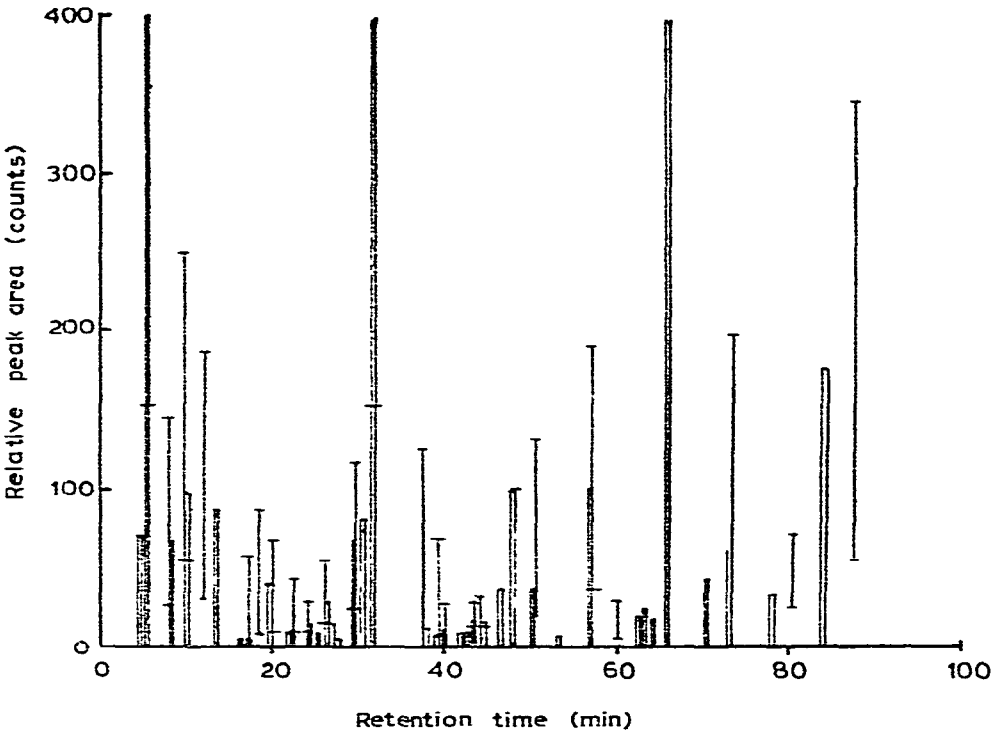

Fig. 7. Diagnostic data for a normal subject.

No.	*Time*	*Height*	*Normal range*	*Remarks*
1	5.59	833.34	152.88–2247.39	N
2	8.46	67.54	24.90– 143.84	N
3	10.20	97.02	53.52– 248.66	N
7	19.91	39.92	– 66.56	N
8	22.87	4.91	– 40.76	N
9	24.44	13.51	8.37– 27.09	N
10	26.56	27.46	14.36– 52.31	N
12	30.73	82.02	22.79– 116.78	N
13	31.76	576.16	152.97–1552.82	N
15	40.08	10.64	6.90– 24.97	N
17	44.69	15.92	10.95– 27.70	N
18	43.62	18.12	11.72– 30.39	N
19	46.45	36.50	– 589.80	N
21	50.41	37.41	– 134.01	N
22	57.16	102.16	33.97– 190.90	N
24	66.04	682.96	– 969.98	N
35	73.41	61.48	– 196.68	N

correlation, as shown in Fig. 9. Consequently, it is felt that the creatinine clearance can be determined by the chromatographic analysis of urine, without collection of blood, which is advantageous with children and serious cases.

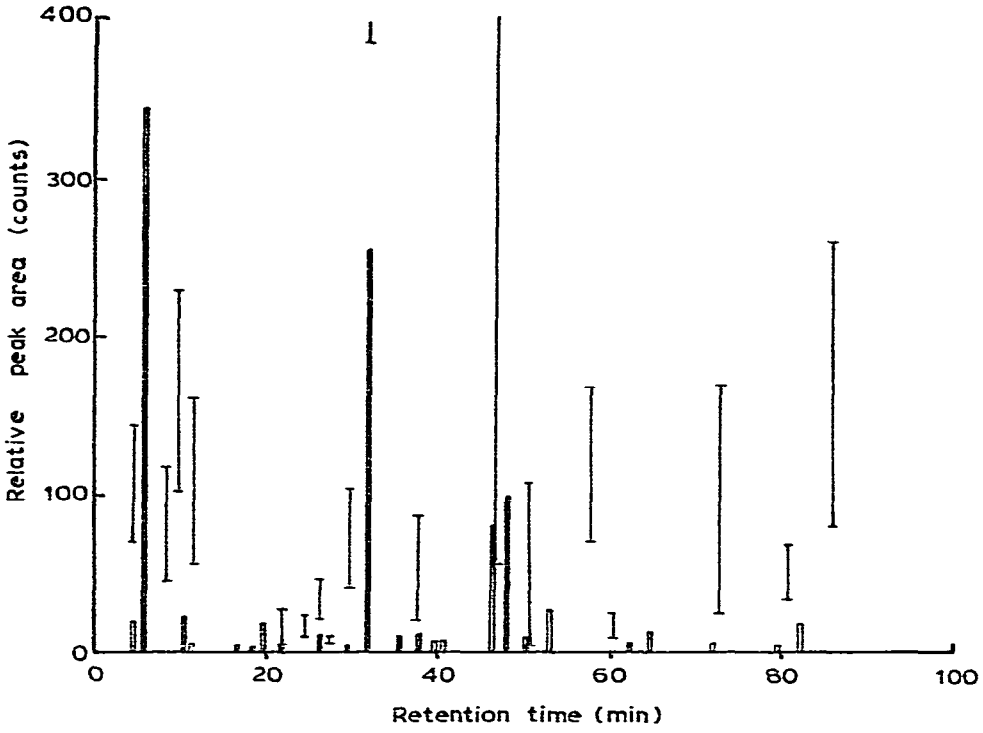

Fig. 8. Diagnostic data for a kidney patient.

No.	*Time*	*Height*	*Normal range*	*Remarks*
4	10.31	22.52	101.79– 228.32	–AA
8	26.29	11.26	20.68– 45.98	–A
11	31.76	254.33	386.28–1319.51	–A
13	46.32	81.31	56.24– 492.47	N
19	71.86	5.25	23.83– 169.94	–A
20	79.35	3.39	33.39– 68.75	–AA

Correlations of peak area between two different peaks are shown in Fig. 10. Whereas a very good correlation between peaks 11 and 135 was obtained for normal subjects (correlation factor 0.955), patients with kidney disorders, such as chronic nephritis, renal insufficiency and diabetes mellites did not conform to this relationship.

Beardmore and Kelley[12] reported that metabolic processes could be estimated by the anion-exchange chromatography of urinary ultraviolet-absorbing compounds. According to their proposals, two constituents shown in Fig. 10 might be metabolically related compounds excreted in urine. Finally, it is felt that diagnosis based on the correlation between two appropriate peaks is most promising for analysis of kidney functions.

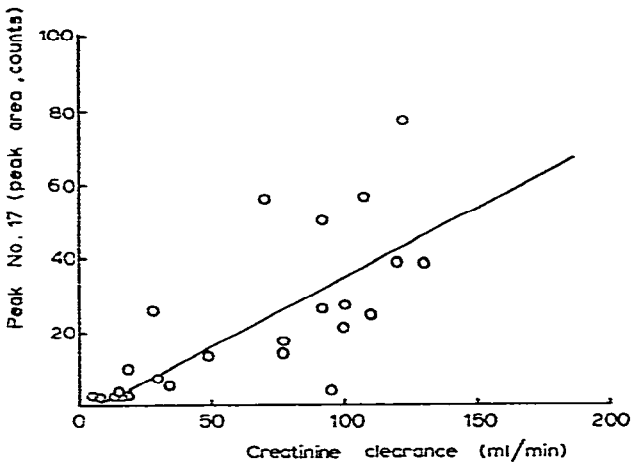

Fig. 9. Correlation between creatinine clearance and chromatographic peak (peak 17). $y = 0.372x - 2.42$; $r = 0.753$; $n = 24$.

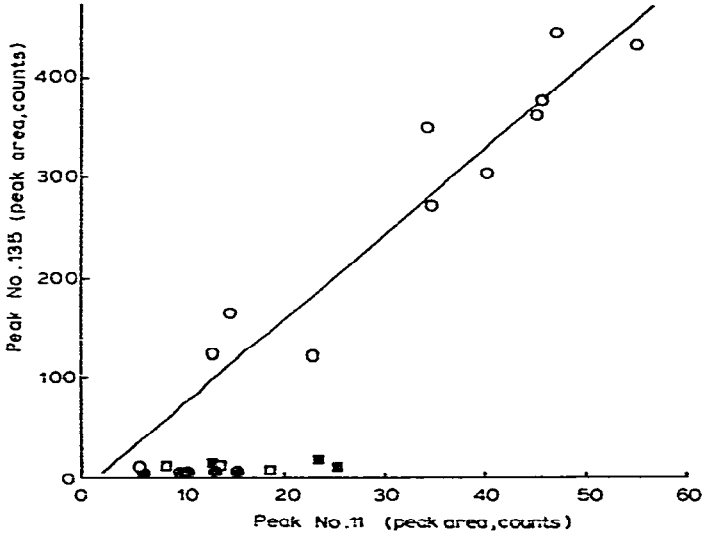

Fig. 10. Correlation between peaks 11 and 135. ○, Normal subject; ■, diabetes mellitus; □, renal insufficiency; ●, chronic nephritis. $y = 8.59x - 12.23$; $r = 0.955$; $n = 11$ (for normal subject).

CONCLUSION

We have demonstrated the performance of a clinical liquid chromatograph consisting of an automated liquid chromatograph combined with a computer for diagnosis. This on-line type of apparatus has potential applications in routine clinical analysis, because of the short analysis time, high resolution and automated diagnostic functions. Variations of the apparatus are possible. Although only a small range of application with respect to diagnostic power was described in this paper, much useful information on body function could be obtained with the apparatus. Its capabilities will be extended further by accumulating pathological data.

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