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Column-switching liquid chromatographic method for the simultaneous determination of iothalamic acid and creatinine in biological fluids

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ABSTRACT

A column-switching liquid chromatographic method for the simultaneous determination of iothalamate and creatinine in human serum and urine was developed. Iothalamate and creatinine were separated on a weakly acidic ion-exchange column (C1) by ion-exclusion chromatography and iothalamate excluded from the column was purified by gel chromatography on a hydrophilic gel column (C2) and then by ion-exchange chromatography on a weakly basic ion-exchange column (C3). Creatinine that was eluted from C1 after iothalamate was transferred to a hydrophilic gel column (C4) and then to a strongly acidic ion-exchange column (C5). The mobile phase for C1–C4 was a pH 3.8 propionate buffer (propionic acid–NaOH = 0.35 + 0.035 mol/kg in water) and a pH 5.6 propionate buffer (propionic acid–NaOH = 0.04 + 0.035 mol/kg in water) was used for C5. Diluted serum and urine samples could be injected directly on to C1, as the matrix of C1 is hydrophilic and C1 is backflushed after the transfer of the creatinine fraction from C1 to C4. Iothalamate and creatinine in the eluates were determined by measuring their ultraviolet absorption at 245 and 234 nm, respectively. The precision (R.S.D.) of the chromatographic method was 1.6% ($n = 7$) and 0.36% ($n = 6$) for diluted serum and urine with iothalamate concentrations of 1.0 and 10.0 μmol/l, respectively, and 0.85% ($n = 7$) and 0.55% ($n = 7$) for diluted serum and urine with creatinine concentrations of 5.77 and 272 μmol/l, respectively.

INTRODUCTION

[^{125}I]iothalamate clearance has been used for the determination of the glomerular filtration rate as an alternative of inulin clearance [1–3]. However, with radioactive compounds there are problems of discarding radioactive waste and the administration of radioactive materials to humans. To avoid these problems, high-performance liquid chromatographic (HPLC) methods for the determination of non-radioactive iothalamate in human serum and urine have been developed [4–8]. Most of these methods required sample pretreatment such as deproteinization or extraction with an organic solvent. Chromatographic systems utilized for the determination of serum creatinine include ion-exchange [9–16], reversed-phase [17,18] and ion-pair chromatography [19] with pretreatment such as deproteinization. Analyses of serum

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creatinine by multi-dimensional HPLC have also been reported [9,17,18,20]. Urinary creatinine has been determined by ion-exchange [10,13], ion-pair [19,21] and reversed-phase chromatography [22].

We have developed a method for on-line sample pretreatment based on ion-exclusion chromatography. The method has been applied to the determination of catecholamines [23], vanillylmandelic acid [24], homovanillic acid [24] and iothalamate [25] in biological fluids. In this paper, the simultaneous determination of iothalamate and creatinine in human serum and urine is described.

Iothalamate in a sample is excluded from a column of a weakly acidic ion exchanger with a hydrophilic matrix (C1) and the excluded iothalamate is switched to a column of hydrophilic gel and then to a column of anion exchanger using a sodium propionate buffer as the mobile phase. Creatinine eluted from C1 after iothalamate is purified further by gel and cation-exchange chromatography by column switching. As the same mobile phase is used for filtration of a sample through C1 and for backflushing of C1 after the transfer of creatinine from C1 to a hydrophilic gel column, C1 could be used repeatedly and the whole process could be automated.

EXPERIMENTAL

Materials

DIP Conray injection, a solution for drip infusion pyelography, containing 50.07 g of iothalamic acid, 3-(acetylamino)-2,4,6-triiodo-5-[(methylamino)carbonyl)]benzoic acid, in the form of N-methyl-D-glucamine iothalamate in 220 ml of the solution, was purchased from Daiichi Seiyaku (Tokyo, Japan) and creatinine of special grade was purchased from Wako (Osaka, Japan). Other chemicals were of analytical-reagent grade from Yashima Pharmaceutical (Osaka, Japan).

Stock standard solutions of iothalamate and creatinine (1 and 10 mmol/l, respectively) were prepared in 0.01 mol/l propionic acid solution and were diluted with mobile phase A described below to give working standard solutions of various concentrations.

Apparatus

The liquid chromatographic system equipped with automatic column-switching valves consisted of four Model 880-PU constant-flow pumps (Jasco, Tokyo, Japan), a Model SP-024-2 dual-head pump (Jasco), a Model KSST-60J automatic injector (Kyowa Seimitsu, Tokyo, Japan), five columns [Asahipak ES-502C, a weakly acidic ion-exchange column, 10 × 0.76 cm I.D.; Asahipak GS-320H, a hydrophilic gel column, 25 × 0.76 cm I.D.; Asahipak ES-502N, a weakly basic ion-exchange column, 10 × 0.76 cm I.D.; Asahipak GS-220H, a hydrophilic gel column, 25 × 0.76 cm I.D.; all of particle size 9 ± 0.5 μm (Showa Denko, Tokyo, Japan); and Dowex 50W-X8, a strongly acidic ion-exchange column, 5 × 0.46 cm I.D., particle size 20–30 μm (Dow Chemical, Midland, MI, USA)], a Model 821-09 automatic six-way valve (Jasco), four Model MVA-4U7H automatic four-way valves (Sanuki Kohgyo, Tokyo, Japan), two Model 870-UV spectrophotometers (Jasco) and two Model RC-125 recorders (Jasco). These components were assembled as shown in Fig. 1.

The sample injection and switching events were controlled by using nine timer units (T1–T9). The first timer unit (T1), controlling the automatic injector and a four-way valve (V4), is equipped with a Model KS-1500 programmable timer (Koizumi Computer, Kobe, Japan), which repeats on and off modes of electric supply (100 V a.c.), at preset time intervals, to the relay circuit of T1 and to those of T2–T9. Each timer unit of T2–T9 is equipped with a motor timer with a maximum graded time of 6, 12 or 30 min (Models SYS-6M, -12M and -30M; Omron Electronics, Kyoto, Japan), and also with a relay circuit. When the preset time of the motor timer is over, the 100 V a.c. supply to the motor timer is shut off and the circuit is reset when the supply of 100 V a.c. from T1 is off. Timer units T2 and T3 control valve V2, T4 controls V4, T5 controls V1, T6 and T7 control V3 and T8 and T9 control V5 (Table I).

Mobile phases

A pH 3.8 propionate buffer (propionic acid–NaOH = 0.35 + 0.035 mol/kg in water) (mobile phase A) was used as the mobile phase for C1–C4, and a pH 5.6 propionate buffer (propi-

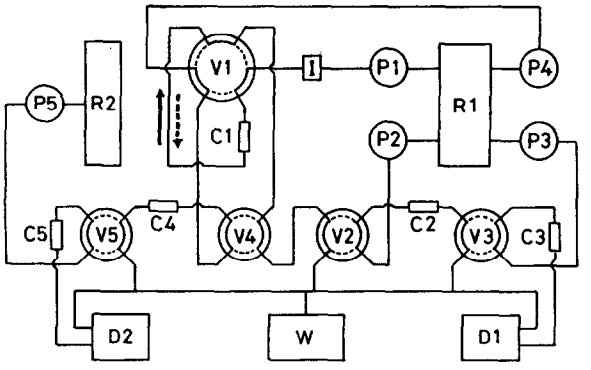

Fig. 1. Diagram of the column-switching equipment. R1 and R2 = mobile phase reservoir containing mobile phase A and B, respectively; I = automatic injector; P1–P3 and P5 = 880-PU; P4 = dual-head pump; C1 = Asahipak ES-502C column (10 × 0.76 cm I.D., 30°C); C2 = Asahipak GS-320H column (25 × 0.76 cm I.D., 50°C); C3 = Asahipak ES-502N column (10 × 0.76 cm I.D., 50°C); C4 = Asahipak GS-220H column (25 × 0.76 cm I.D., 30°C); C5 = Dowex 50W-X8 column (5 × 0.46 cm I.D., 40°C, packed in the laboratory); V1 = six-way automatic valve (full line and broken line represent rotor positions B and A of the valve, respectively); V2–V5 = four-way automatic valve (full line and broken line represent rotor positions L and R, respectively; D1 and D2 = spectrophotometers measuring absorbance at 245 and 234 nm, respectively; W = waste. When the rotor position of V1 is B and that of V2–V5 is L, mobile phases flow through the channels drawn with full lines, and when the rotor position of V1 is A and that of V2–V5 is R, mobile phases flow through the channels drawn with broken lines. The arrow drawn with a full line indicates the direction of flow of mobile phase A pumped by P1 when the rotor position of V1 is B. When the rotor position of V1 is A, mobile phase A pumped by P1 and P4 passes through the channels of V1 drawn with a broken line, and the mobile phase A pumped by P4 flows in the direction indicated by the arrow drawn with a broken line.

onic acid–NaOH = 0.04 + 0.035 mol/kg in water) (mobile phase B) was used for C5. Water of ultra-pure grade, obtained by using reverse osmosis (ROpure 40, Barnstead, Boston, MA, USA), ion-exchange and charcoal adsorption (NANOpure II, Barnstead) in series, was used to prepare mobile phases. They were filtered through a membrane filter (Type HV, pore size 0.45 μm; Millipore, Bedford, MA, USA) and degassed before use. The flow-rate of these mobile phases was 1.2 ml/min.

Sample preparation

Samples in plastic vials were kept frozen at −70°C and thawed before use. Serum (0.2 ml) was mixed with 1.8 ml of 2.5% (w/v) propionic acid solution. Urine (0.2 ml) was mixed with 1.8 ml of 2.5% propionic acid solution and then mixed with 3 ml of mobile phase A. The diluted sample was filtered through a disposable membrane filter (Shodex DT MX-13K, pore size 0.2 μm; Showa Denko) and poured into a vial. It was placed in the rack of the automatic injector and analysed within 10 h after preparation. For the determination of recovery, one volume of serum was diluted with nine volumes of 2.5% propionic acid solution containing iothalamate and creatinine to give a diluted serum sample with an iothalamate concentration of 1.0 μmol/l and creatinine concentration increased by 5.0 μmol/l, and one volume of urine was diluted with nine volumes of 2.5% propionic acid solution containing iothalamate and creatinine and fifteen volumes of mobile phase A to give a diluted urine sample with an iothalamate concentration of 10.0 μmol/l and creatinine concentration increased by 300 μmol/l (Table II).

Column switching and detection

The rotors of valves V1–V5 were positioned at B and L (full line in Fig. 1). Mobile phase A pumped by P1 flowed through the cation-exchange column (Asahipak ES-502C, C1) to waste, and mobile phase A pumped by P4 flowed through the hydrophilic gel column (Asahipak GS-220H, C4). When the switch of the programmable timer of T1 was turned on, T2–T9 came on, and sample (300 μl) was injected on to C1 from which anionic compounds together with iothalamate were excluded. The iothalamate fraction was transferred from C1 to Asahipak GS-320H (C2) via V2 by column switching. Next, creatinine was transferred to Asahipak GS-220H (C4) by switching the rotor position of V4 to R. When the transfer of creatinine fraction to C4 was over, the rotor position of V1 was changed to A to disconnect C1 and C4, and at the same time to backflush C1 by the mobile phase A pumped by P4. The creatinine fraction transfered to C4 was eluted with mobile phase A pumped by P1 and transferred to the Dowex 50W-X8 column (C5) via V5. The iothalamate fraction was transferred from C2 to Asahipak ES-502N (C3) via V3 and eluted from C3 with mobile phase A pumped by P3. Creatinine was eluted from C5 with mobile phase B pumped by

TABLE I

TIMING AND SEQUENCE OF EVENTS OF AUTOMATED COLUMN SWITCHING FOR THE DETERMINATION OF IOTHALAMATE AND CREATININE

Time (min)	On–off of timers of the timer units									Rotor position of valves					Event
	T1	T2	T3	T4	T5	T6	T7	T8	T9	V1	V2	V3	V4	V5	
0	On	On	On	On	On	On	On	On	On	B	L	L	L	L	Sample injection on to C1
3.8	On	Off	On	On	On	On	On	On	On	B	R	L	L	L	Connection of C1 and C2. Transfer of iothalamate fraction from C1 to C2
5.7	On	Off	Off	On	On	On	On	On	On	B	L	L	L	L	Disconnection of C1 and C2. End of transfer of iothalamate fraction from C1 to C2
6	On	Off	Off	Off	On	On	On	On	On	B	L	L	R	L	Connection of C1 and C4. Transfer of creatinine fraction from C1 to C4
8	On	Off	Off	Off	Off	On	On	On	On	A	L	L	R	L	Backflushing of C1 with P4. End of transfer of creatinine fraction from C1 to C4
11.6	On	Off	Off	Off	Off	On	On	Off	On	A	L	L	R	R	Connection of C4 and C5. Transfer of creatinine fraction from C4 to C5
14	On	Off	Off	Off	Off	On	On	Off	Off	A	L	L	R	L	Disconnection of C4 and C5. End of transfer of creatinine fraction from C4 to C5
14.2	On	Off	Off	Off	Off	Off	On	Off	Off	A	L	R	R	L	Connection of C2 and C3. Transfer of iothalamate fraction from C2 to C3
17.5	On	Off	Off	Off	Off	Off	Off	Off	Off	A	L	L	R	L	Disconnection of C2 and C3. End of transfer of iothalamate fraction from C2 to C3
47	Off	Reset	Reset	Reset	Reset	Reset	Reset	Reset	Reset	B	L	L	L	L	End of blackflushing of C1
55	On	On	On	On	On	On	On	On	On	B	L	L	L	L	Injection of next sample

TABLE II

RECOVERY OF IOTHALAMATE AND CREATININE ADDED TO DILUTED SERUM AND URINE

Sample	Analyte	Concentration of analyte added (μmol/l)	Recovery (%)[a]
Serum (10-fold dilution)	Iothalamate	1.0	100.2 ± 1.6
	Creatinine	5.0	102.1 ± 2.19
Urine (25-fold dilution)	Iothalamate	10.0	100.5 ± 0.36
	Creatinine	300	99.6 ± 1.78

[a] Mean ± R.S.D. ($n = 7$).

P5. After 47 min from sample injection, the programmable timer of T1 was off, T2–T9 were reset, the rotor position of V1 became B and backflushing of C1 ended, and at the same time the rotor position of V4 became L. Eight minutes later, the timer of T1 was on, and next sample was injected (Table I). Iothalamate and creatinine were determined from their peak heights on the chromatogram obtained with ultraviolet detection at 245 and 234 nm, respectively.

RESULTS AND DISCUSSION

Sodium propionate buffers were used as the mobile phases because they are stable, easy to

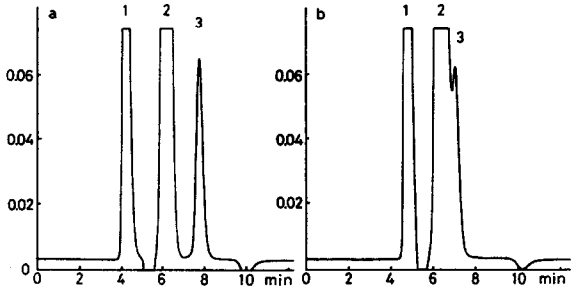

Fig. 2. Elution of (1) iothalamate, (2) uric acid and (3) creatinine from C1. (a) Elution pattern from a new column and (b) from the column after 70 serum samples had been injected. Ordinate, absorbance at 245 nm; abscissa, retention time.

prepare and do not allow microorganisms to grow. Among the propionate buffers of various concentrations and pH tried, mobile phase A gave the best result for the purification of iothalamate. As iothalamate is anionic and creatinine is cationic at pH 3.8, they were separately eluted from the weakly acidic cation-exchange column (C1) as shown in Fig. 2. Iothalamate could be separated from most impurities by filtration through the hydrophilic gel column (C2), and after elution from C3 no endogenous UV-absorbing peak was found at the retention time of iothalamate in the elution patterns of blank serum and urine samples (Fig. 3). Most impurities present in the creatinine fraction eluted from C1 could be removed by filtration through the hydrophilic gel column (C4), and the creatinine fraction eluted from C4 was transferred to C5 as shown in Fig. 4. The creatinine fraction transferred from C4 to C5 could not be eluted with mobile phase A, as the ion-exchange capacity of Dowex 50W-X8 column is higher than that of Asahipak ES-502C (C1).

With the use of the mobile phase of pH 5.6, in which the net charge of creatinine is decreased, elution of creatinine was successful (Fig. 5). Creatine was eluted from C1 with iothalamate and did not interfere with the determination of creatinine. The rate of conversion of creatine to creatinine in aqueous solution is maximum at pH 4 [26]. However, the conversion was 0.9% after 10 h when a solution of creatine in mobile phase A at concentration of 200 μmol/l was incubated at 20°C. As the concentration of creatine in serum is of the same order as that of creatinine and in urine is much lower [27], the increase in the concentration of creatinine in diluted serum and urine samples before analysis due to the dehydration of creatine present in diluted samples will be less than 1% of the concentration of creatinine in diluted samples.

The change in the efficiency of C1 was checked after 70 injections of serum samples on to C1. The retention time of iothalamate increased and that of creatinine decreased, as shown in Fig. 2b. However, after the timing of the switching of valves was adjusted, separation of iothalamate and creatinine from other UV-

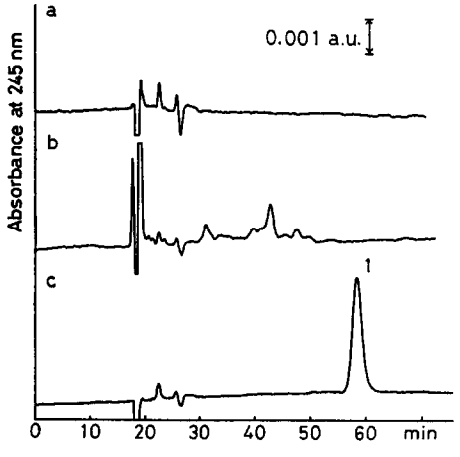

Fig. 3. Elution of samples from C3. (a) Diluted serum (10-fold dilution, (b) diluted urine (25-fold dilution) and (c) standard solution of iothalamate (peak 1, 1.0 μmol/l). Ordinate, absorbance at 245 nm; abscissa, retention time.

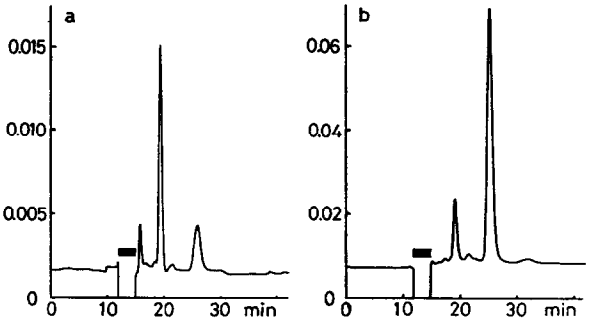

Fig. 4. Elution from C4 of the creatinine fraction transferred from C1 to C4. (a) Diluted serum sample and (b) diluted urine sample. Ordinate, absorbance at 245 nm; abscissa, retention time. The black horizontal bars represent the creatinine fractions transferred from C4 to C5.

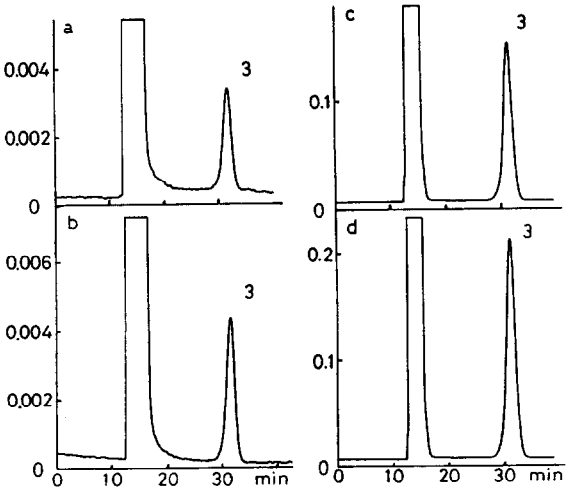

Fig. 5. Elution of creatinine (peak 3) from C5. (a) Standard solution (4.0 μmol/l); (b) diluted serum sample (calculated concentration 5.77 μmol/l); (c) standard solution (200 μmol/l); (d) diluted urine sample (calculated concentration 272 μmol/l). Ordinate, absorbance at 234 nm; abscissa, retention time.

absorbing peaks was satisfactory, as shown in Figs. 3, 5 and 6. Adjustment of the times of switching of the valves is as follows. First, the switching times of V2, V4 and V1 are adjusted so that the peaks of iothalamate and creatinine

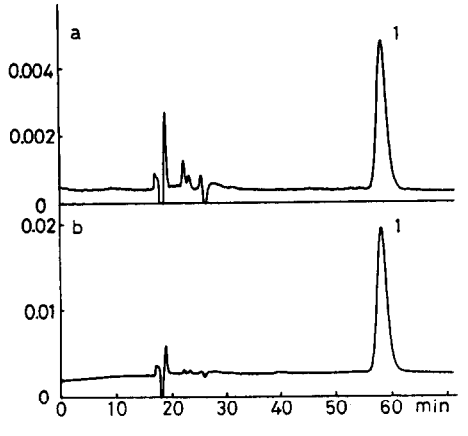

Fig. 6. Elution of samples containing iothalamate (peak 1). (a) Diluted serum sample prepared from serum taken from a patient 150 min after intravenous administration of 1.0 ml of DIP Conray injection containing 227.6 mg (388.5 μmol) of iothalamate (calculated concentration 1.31 μmol/l); (b) diluted urine sample prepared from urine collected from the same patient between 135 and 165 min after the intravenous administration of iothalamate (calculated concentration 5.03 μmol/l). Ordinate, absorbance at 245 nm; abscissa, retention time.

disappear from the chromatogram monitoring the eluate from C1. Then the switching times of V3 and V5 are adjusted so that the peaks of iothalamate and creatinine in the chromatograms monitoring the eluates from C2 and C4 disappear. Chromatograms showing the transfer of creatinine fraction from C4 to C5 are shown in Figs. 4a and b.

The sensitivity of this method was high enough to determine the concentration of iothalamate and creatinine in diluted serum and urine samples. The limit of detection was 0.1 μmol/l for iothalamate and 0.3 μmol/l for creatinine at a signal-to-noise ratio of 3. the relationship between the peak height (x) and the concentration of the analyte (y) was linear. In the ranges 0.5–20 μmol/l for iothalamate and 2–400 μmol/l for creatinine in mobile phase A, the equations were $y = 0.302x - 0.0155$ ($r = 0.9999$) and $y = 1.313x + 0.383$ ($r = 0.9999$), respectively.

The recovery of iothalamate and creatinine added to serum and urine is shown in Table II. The within-day relative standard deviation (R.S.D.) for iothalamate in diluted pooled serum (1.0 μmol/l of iothalamate) was 1.6% ($n = 7$) and in diluted pooled urine (10.0 μmol/l) it was 0.36% ($n = 6$). The within-day R.S.D. for creatinine in diluted pooled serum (calculated concentration 5.77 μmol/l) was 0.85% ($n = 7$) and in diluted pooled urine (calculated concentration 272 μmol/l) it was 0.55% ($n = 7$).

The creatinine concentration obtained by the present method (y) was compared with that obtained by the modified Jaffé method [28] (rate

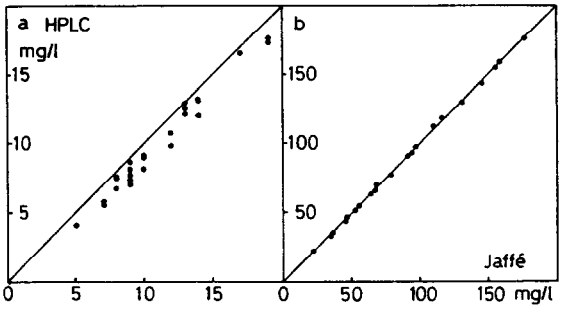

Fig. 7. Comparison between the concentration of creatinine in serum determined by the present method and Jaffé's method. Ordinate, concentration determined by the present method (y); abscissa, concentration determined by Jaffé's method (x). (a) Lower and (b) higher concentration ranges. The diagonal line represents $x = y$.

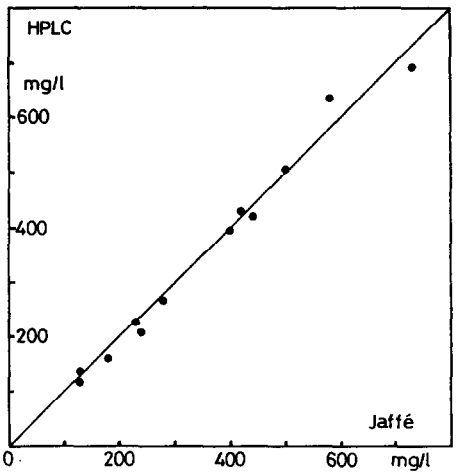

Fig. 8. Comparison between the concentration of creatinine in urine determined by the present method and Jaffé's method. Ordinate, concentration determined by the present method (y); abscissa, concentration determined by Jaffé's method (x). The diagonal line represents $x = y$.

assay) (x). In the concentration range 5–19 mg/l, the serum creatinine value obtained by the present method was lower than that obtained by the Jaffé method. The regression line was $y = 1.005x - 1.22$ ($n = 29$, $r = 0.9879$) and in the higher concentration range of 22–177 mg/l, $y = 1.0013x - 0.724$ ($n = 21$, $r = 0.9997$) (Fig. 7). The urinary creatinine concentration obtained by the present method was also lower than that of Jaffé method, $y = 1.0175x - 10.4$ ($n = 12$, $r = 0.9916$) (Fig. 8).

These results indicate that this column-switching liquid chromatographic method is suitable for the determination of creatinine and iothalamate in human serum and urine.

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