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A Rapid Microassay for Dichloroacetate in Serum by Gel-Permeation Chromatography

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Summary: We have developed a novel, rapid microassay for dichloroacetate in the serum. The serum sample is directly injected into a gel-permeation high-performance liquid chromatography apparatus. The peak of dichloroacetate appears after a giant protein peak. The method requires a very small amount of serum (10 µl), and the analysis time is short (20 min). Using this micro method, we measured the serum concentrations of dichloroacetate in healthy adult volunteers and paediatric patients with congenital lactic acidosis. Although the effect of dichloroacetate on the neurological manifestations of congenital lactic acidosis has not been proved to be beneficial, the potential usefulness of dichloroacetate in refractory lactic acidosis in cardiac and respiratory failure has been recognized, and human as well as animal studies have been undertaken in many laboratories. To prevent possible side effects of dichloroacetate, it has been recommended that the minimal effective dose be used. Our microassay method is useful for both human and animal experiments, even after administration of minimal doses.

Introduction

Dichloroacetate as the sodium salt has been used for the treatment of hyperglycaemia, lactic acidosis, and hypercholesterolaemia (1–7). Although dichloroacetate has many metabolic effects on glucose, lipid and amino acid metabolism, its main pharmacologic effect has been shown to be the activation of the pyruvate dehydrogenase[1]) complex by inhibiting pyruvate dehydrogenase kinase[1]) (7–11).

The clinical usefulness of dichloroacetate has been hampered by its possible toxic and mutagenic effects (7). Peripheral neuropathy and testicular atrophy have been reported in humans as well as animals after chronic administration of dichloroacetate (12). Recently, however, dichloroacetate has been reevaluated in response to the many reports on its efficacy in the refractory acidosis encountered in emergency medicine, such as cardiac arrest and septic shock (13–15).

Although there have been few reports on the acute toxicity of dichloroacetate, it has been recommended that the minimal effective dose be used in order to prevent possible adverse effects (7). Dichloroacetate has been measured by gas-chromatography (11), but the measurement is time-consuming and requires 2 ml of blood. Because of these technical difficulties, blood concentrations of dichloroacetate have not been routinely measured.

In the present study, we have developed a rapid micromethod for dichloroacetate measurement using gel permeation chromatography. With this micromethod, we have investigated the pharmacokinetics of dichloroacetate by measuring its level in serum in various patients with lactic acidosis, as well as in healthy adult volunteers. Since the new method requires a very small amount of serum (10 µl), and needs no extraction procedure for sample preparation, it is suitable for measuring serum concentrations of dichloroacetate in human as well as animal experiments.

Materials and Methods

Dichloroacetate (sodium salt) was purchased from Tokyo Kasei (Tokyo). The other chemicals were of the highest quality available and obtained from standard sources.

[1]) Enzymes:
Pyruvate dehydrogenase, EC 1.2.4.1; Pyruvate dehydrogenase kinase, EC 2.7.1.99.

Blood samples were drawn from patients with lactic acidosis of various actiologies and from normal adult volunteers. The basic disorders of the patients and doses of dichloroacetate are summarized in table 1. Informed consent was obtained from the parents of the patients before dichloroacetate administration. The volunteers were given 35 mg/kg of dichloroacetate orally. Blood samples were drawn before and after oral dichloroacetate loading at 30 minutes intervals. Serum was separated immediately and kept frozen at −20 °C until analysis.

A small amount (15 to 25 µl) of serum was directly injected into a high-performance liquid chromatography (HPLC) apparatus. A gel-permeation HPLC column (Asahipak GS-320, Asahi Kasei, Tokyo) was equipped with a pump (Waters, Model 6000A) and UV spectrophotometer (Soma UV detector, Soma Kogaku, Tokyo). The column was eluted with 0.01 mol/l ammonium acetate (pH 4.0)/acetonitrile (9+1, by vol.) at a flow rate of 2.0 ml/min, and the eluate was monitored at 220 nm. The quantitation of dichloroacetate was carried out by comparing the peak height of dichloroacetate with the calibration curve constructed by plotting the peak heights of various known amounts of dichloroacetate.

Dichloroacetate was also measured by gas-chromatography according to the method of *Wells* et al. (11) with trichloroacetic acid as the internal standard.

Results

The new chromatographic method of dichloroacetate measurement

The chromatogram of normal serum is shown in figure 1a. In the gel-permeation column, molecules whose sizes are greater than M_r 40 000 are excluded from the column bed, and the smaller molecules are retained in the column for various times according to the interaction between the molecules and the packing material. Thus the chromatogram of normal serum consists of a giant peak which represents the serum components with molecular mass greater than 40 000 (mostly proteins) and several unknown peaks following it. The chromatogram of the serum from a volunteer after dichloroacetate intake is shown in figure 1c. As shown in the figures, a distinct peak appeared at 12.2 min. A peak with exactly the same retention time was observed when serum previously supplemented with authentic dichloroacetate was analysed (fig. 1b). The chromatogram of serum from Patient 1 is shown in figure 1d. The calibration curve constructed by injecting various known amounts of authentic dichloroacetate showed a linear relation between 0 to 500 mg/l as shown in figure 2. The minimal measurable concentration was 5 mg/l when 25 µl of serum was injected, but a much lower concentration was measurable by increasing the amount injected, or by increasing the sensitivity of the UV detector. To assess the reproducibility of the method, we injected replicate aliquots of the sample. The coefficient of variation (CV) was found to be very small (CV = 2.4%, n = 8).

We measured the levels of dichloroacetate in 9 serum samples by conventional gas chromatography concomi-

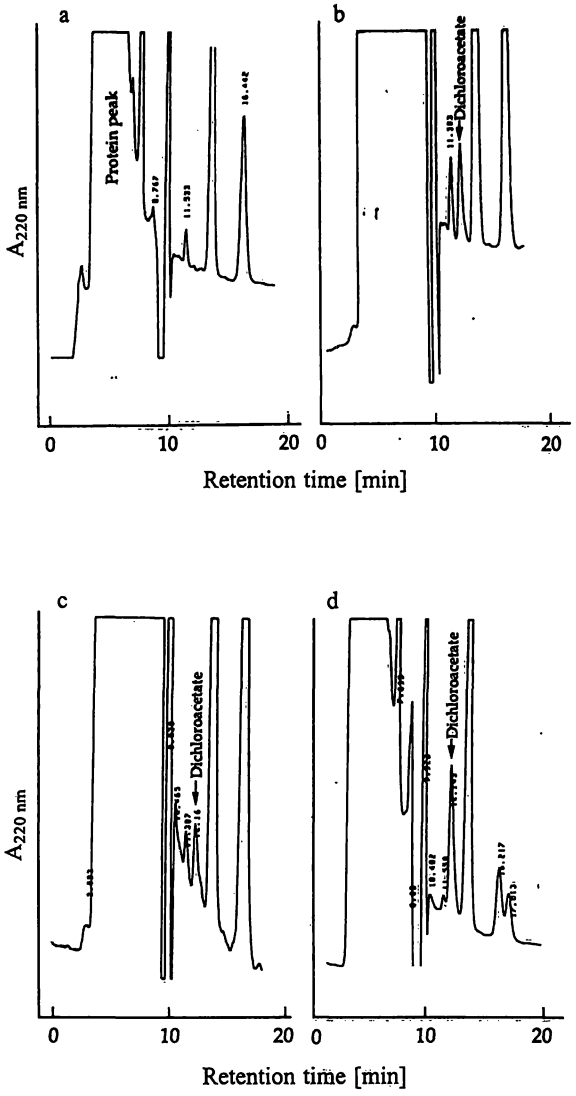

Fig. 1 Chromatograms of dichloroacetate in serum.
(a) Chromatogram of normal serum. Note the giant peak immediately after the solvent peak.
(b) Chromatogram of normal serum mixed with authentic dichloroacetate.
(c) Chromatogram of the serum of a volunteer after a single oral dichloroacetate load.
(d) Chromatogram of the serum from Patient 1.

Tab. 1 Profiles of the patients.

Patient No.	Age (a)	Sex	Diagnosis	Dose of dichloroacetate (mg/kg · d)
1	8 5/12	♀	Pyruvate dehydrogenase complex deficiency	80
2	3 8/12	♂	Pyruvate dehydrogenase complex deficiency	40
3	8 6/12	♀	Mitochondrial encephalopathy, lactic acidosis and stroke-like episode (MELAS)	30
4	9/12	♀	Leigh encephalopathy	30

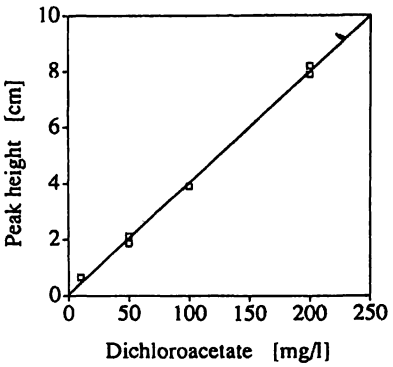

Fig. 2 Calibration curve of dichloroacetate. Serum samples with known concentrations of dichloroacetate (10, 50, 100, 200 mg/l) were prepared by adding authentic dichloroacetate to serum from a healthy adult volunteer. A 25 µl aliquot of each sample was injected into HPLC. Good linearity was obtained between 10 to 200 mg/l (y = 0.04x + 0.045, r = 0.997).

tantly with the new micromethod (11). As shown in figure 3, there was a good correlation between the results obtained from the two methods.

Finally, the specificity of the method was ascertained by injecting the sera from 10 patients who were taking different drugs (antiepileptic drugs, antibiotics) into the HPLC. No peaks were observed around the retention time of the dichloroacetate peak (data not shown).

Pharmacokinetics of dichloroacetate in healthy adult volunteers

Using the micromethod, we investigated the pharmacokinetics of dichloroacetate in healthy adults. The time course of the levels of dichloroacetate in two volunteers is shown in figure 4. The dichloroacetate was absorbed quickly and reached a peak concentration within 30–60 minutes. The level then dropped rapidly, and by 5 hours dichloroacetate had disappared from the serum. The half life of dichloroacetate elimination from the serum was calculated to be around 30 min by the modified method of *Wagner & Nelson* (16).

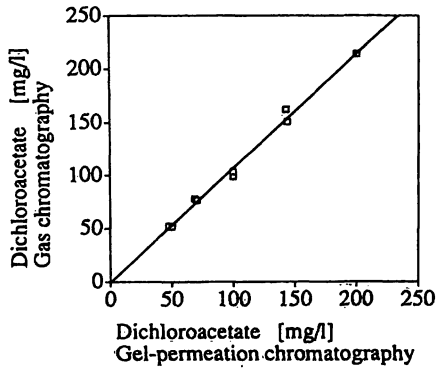

Fig. 3 Correlation between the new micromethod and gas chromatography. A good correlation was obtained between the two methods (y = 1.07x − 0.74, r = 0.992).

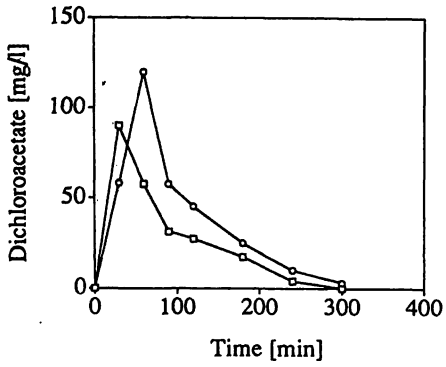

Fig. 4 Concentrations of dichloroacetate in the sera from healthy adult volunteers after a single oral dose of dichloroacetate (35 mg/kg). The half lives of elimination are 0.534 h (−□−) and 0.573 h (−○−).

Measurement of serum dichloroacetate levels in patients

The typical time course of serum dichloroacetate concentrations is shown in figures 5a and 5b. In patients 1, 2 and 3, dichloroacetate had been orally administrated for various periods previously. In Patients 2, 3 and 4, the attained peak levels of dichloroacetate were different,

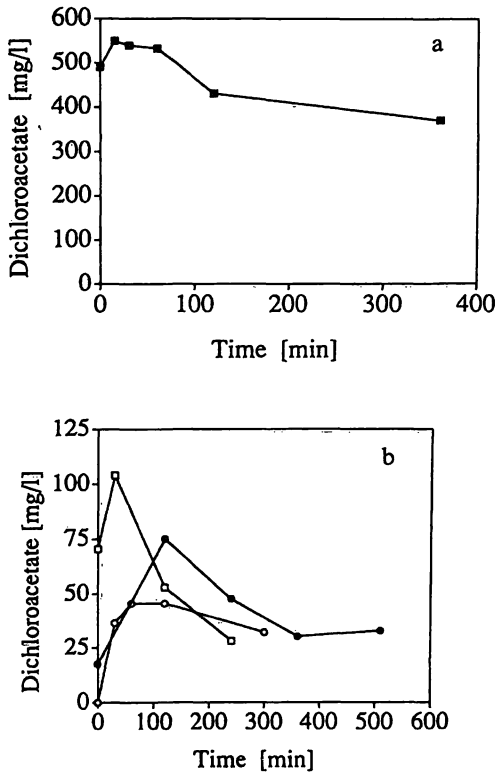

Fig. 5 Concentrations of dichloroacetate in the sera from the patients.
(a) Patient 1 (−■−)
(b) Patients 2 (−□−), 3 (−●−), and 4 (−○−).
In Patients 1, 2 and 3, dichloroacetate had already been administered for various times before the measurements. Thus the levels of dichloroacetate before oral administration (at time 0) were not zero. The half-lives of dichloroacetate for Patients 1, 2, and 3 were 245 h, 0.18 h and 0.87 h respectively.

although the doses of dichloroacetate per body weight were similar. It was also shown that the ratio of dose to blood level was not linear, as indicated by the very high levels of dichloroacetate in Patient 1. The very prolonged half-life of dichloroacetate in Patient 1 suggests an impaired elimination of dichloroacetate from the circulation in this case.

Discussion

We demonstrated that the micromethod for dichloroacetate measurement is useful for monitoring serum dichloroacetate. Once the HPLC apparatus was set up, it usually took only 20 minutes to obtain the results. In the conventional gas-chromatographic method, it would take at least several hours, since deproteinization and derivatization of dichloroacetate must be performed before the chromatographic measurement (11). Addition of internal standard is also required in the gas-chromatographic method. Furthermore, the new method requires much less sample: the gas-chromatographic method requires 1 ml of serum, whereas as little as 10 µl is enough for the micro method. The serum contained in a glass capillary is sufficient for the measurement of dichloroacetate by the new method. We are therefore able to monitor dichloroacetate concentrations even in small animals.

The pharmacokinetics of dichloroacetate determined by the micro method are comparable with the previously reported values obtained with a single intravenous injection (5, 11, 17, 18). Although the accurate calculation of the half-life was difficult when the drug was administrated orally, we estimated the approximate half life of dichloroacetate by a modified calculation method of *Wagner & Nelson* (16). In healthy adults, the half-life was approximately 30 minutes, although the calculated half-life of dichloroacetate was much longer than this in Patient 1. It has been reported that the elimination of dichloroacetate from the circulation follows saturation kinetics (11), and tissue accumulation of dichloroacetate occurs when dichloroacetate is administered for a long time. Adverse effects such as polyneuropathy and testicular atrophy have been reported after chronic dichloroacetate treatment in human and animal studies (7, 12). Considering the unpredictability of exact blood levels of dichloroacetate, it is pertinent to measure them in patients treated with dichloroacetate. The fact that dichloroacetate therapy has not yet been accepted as a standard therapy for lactic acidosis is mainly due to its possible toxic side-effects. It is important to maintain the reported minimum therapeutic level (130 mg/l) of dichloroacetate (11). Although it is undoubtedly effective in lowering blood lactate and pyruvate in patients with congenital lactic acidosis, it has been reported to show little effect in improving existing neurologic symptoms (7). Recently, however, its potential clinical usefulness has been reappraised, especially in critical care medicine (13–15). It has been demonstrated that dichloroacetate is effective in correcting severe acidosis refractory to the conventional sodium bicarbonate administration. It has also been shown that it has a protective effect against myocardial ischaemic damage. To confirm its potential clinical usefulness, many laboratories have been conducting animal experiments to determine its efficacy and safety. Our micromethod is suitable for monitoring blood dichloroacetate concentrations in animal experiments as well as in clinical trials.

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