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Determination of salicylic acid in human serum with capillary zone electrophoresis

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Abstract

The determination of salicylic acid (SA), a metabolite of aspirin, in human serum was developed using capillary zone electrophoresis (CZE) with diode array detection. The reproducibility of separation and quantification with CZE analysis of the extract of SA from human serum was appropriate for the intra- and inter-day assay coefficients. A high correlation was revealed between the serum SA levels in volunteers determined by CZE and those determined by a fluorescence polarization immunoassay ($r=0.973$, $n=12$), although the former values were slightly higher than the latter. There were no peaks interfering with the assay of SA by internal standard method. This CZE method could provide a simple and efficient method for monitoring SA in patients. © 1998 Elsevier Science B.V.

Keywords: Therapeutic drug monitoring; Salicylic acid

1. Introduction

Despite of the development of several new non-steroidal anti-inflammatory drugs, aspirin is still the most widely used analgesic, anti-inflammatory and antipyretic agent. Aspirin produces these properties by covalently acetylating the serin residue at position 530 of cyclooxygenase-1, a constitutive prostaglandin synthetic enzyme, and also at position 516 of cyclooxygenase-2, an enzyme induced in inflammation. Low doses of aspirin (40 to 325 mg) have been used as anti-thrombotic agents because of aspirin's inhibition of thromboxane A_2 production via the selective acetylation of cyclooxygenase in platelets. Orally administered aspirin is absorbed and rapidly hydrolyzed to salicylic acid (SA); the serum half-life of aspirin is only about 15 min. The serum half-life of SA is 2 to 3 h following low-dose consumption of aspirin, about 12 h for the therapeutic doses commonly used for inflammation, and 15 to 30 h following high doses or intoxication.

The relationship between serum SA levels and the therapeutic effects of aspirin has been well documented. Serum SA concentrations of 100 μg/ml and 150 to 300 μg/ml are required for analgesic and anti-inflammatory effects, respectively. When aspirin is frequently used to manage acute rheumatic fever, the serum SA concentration is increased to 300 to

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400 μg/ml. However, symptoms of intoxication may appear at 300 μg/ml level, and severe sequelae of intoxication including tetany and acidosis may occur at levels more than 500 μg/ml [1,2]. Therapeutic drug monitoring is therefore necessary for the treatment of rheumatoid arthritis with aspirin. Aspirin also has a relatively high risk of inducing poisoning following improper use, especially in young children. Monitoring of the serum SA level may be essential in decisions regarding the treatment of alkaline diuresis or hemodialysis in case of severe aspirin intoxication. On the other hand, the optimal doses of aspirin for producing selective antiplatelet action have not been well established, although an inhibition of platelet aggregation has been known to correlate with the area under the curves (AUCs) of the serum level of aspirin [3]. Thus, monitoring of serum aspirin and SA levels should be also considered for exploring such optimal doses of aspirin.

SA in serum can be measured in clinical laboratories with a fluorescence polarization immunoassay (FPIA), which is the most common method among the various immunoassays [4]. However, an FPIA is not suitable for the simultaneous assay of several drugs and/or metabolites, and shows cross-reactivities to their analogs or metabolites such as gentisic acid (GA). The major metabolic pathways of SA are conjugation with glucuronic acid or glycine (salicyluric acid; SU), and oxidation to GA. High-performance liquid chromatography (HPLC) is an alternative to the immunoassays for the determination of salicylates, and can analyze metabolites simultaneously [5–7]. Some disadvantages of this method are the troublesome and time-consuming pretreatment (e.g., deproteinization) of body fluids, high running cost due to expensive separation columns, and the use of a large volume of organic solvent, which is environmentally unsound. Capillary electrophoresis (CE) has become an attractive approach for the analysis of pharmaceuticals. The benefits of CE, including the simultaneous measurement of several drugs, specificity of separation, low running cost and the avoidance of organic buffers are favorable for the determination of serum drug concentrations [8–10]. To facilitate the therapeutic drug monitoring of aspirin, the present study was designed to determine the SA levels in human serum using capillary zone electrophoresis (CZE).

2. Experimental

2.1. Reagents

The standard drugs used were as follows: aspirin, sodium salicylate, diisopropyl fluorophosphate (DFP) and eserine sulfate were from Wako Pure Chemical Industries (Osaka, Japan). SU and GA (sodium salt hydrate) were from Sigma (St. Louis, MO, USA). 3-Isobutyl-1-methylxanthine was from Aldrich (Milwaukee, WI, USA). Drug-free human serum was from Bio-Rad Labs. (Anaheim, CA, USA). All other reagents of analytical grade were purchased from Kanto (Tokyo, Japan) and Wako.

2.2. CZE and FPIA

The CZE experiments were performed on an HP^{3D} CE system with a diode array UV detection system (Hewlett-Packard, Wilmington, DE, USA). HP^{3D} CE ChemStation software was used for instrumental control, data acquisition and data analysis. In all experiments, a constant voltage of 25 kV was applied, the temperature was set at 30°C, and the detection was made at 210 nm.

A fused-silica extended light path capillary (64.5 cm×75 μm I.D., Hewlett-Packard) was used for all CZE separations. The extended light path capillary, which is also called a bubble cell capillary, had an internal diameter of 225 μm at the detector located 56 cm from the inlet end [11]. The composition of the electrophoresis running buffer was 100 m*M* boric acid (pH 8.8 adjusted with 1 *M* NaOH), which was filtered through a Millipore filter (Type HV, 0.5 μm) and ultrasonically degassed before use. The capillary was rinsed with 0.1 *M* NaOH for 2 min, and then with running buffer for 3 min before each analysis. A sample was injected into the capillary by the vacuum system at 50 mmHg (1 mmHg=133.322 Pa) for 20 s (injection volume: almost 100 nl).

The FPIA assay was performed using a TDx analyzer (Dainabot Labs., Tokyo, Japan).

2.3. Stability of aspirin in serum

Human serum spiked with 100 μg/ml aspirin was incubated at 37°C in the absence and presence of 0.5 m*M* diisopropyl fluorophosphate (DFP) or 10 m*M*

eserine sulfate. Samples at 5 min, 30 min, 1 h, 2 h, 3 h, 5 h and 8 h after the start of incubation were analyzed by CZE.

2.4. Preparation of standard solution and serum samples

Stock solutions of aspirin, SA, SU and GA were prepared in deionized water or methanol and stored at 4°C. For the study of reproducibility and recovery, various concentrations of solution were prepared by diluting the stock solution with deionized water or human serum just before analysis. In only the assay for the comparison of the CZE and FPIA methods, a TDx calibrator for SA (Dainabot Labs.) was used for the calibration. The samples from 12 healthy volunteers ($n=10$, 2 h and 5 h after a single oral 660 mg aspirin administration; $n=2$, 2 h and 3 h after a single oral 990 mg aspirin administration) and a rheumatoid arthritis patient were centrifuged at 3000 *g* for 10 min and incubated overnight at 37°C. A 100-μl aliquot of each serum sample was vortex-mixed with 150 μl of acetonitrile containing an internal standard (I.S.: 3-isobutyl-1-methylxanthine, 50 μg/ml) and centrifuged at 13 400 *g* for 2 min.

3. Results and discussion

3.1. Determination of SA with CZE

Fig. 1B is a typical electropherogram of the pretreated human serum spiked with standard drugs including aspirin, SA, SU and GA. Under the described conditions, these substances were well separated within 8 min. Acetonitrile used for deproteinization produces a solute-stacking effect allowing the injection of a larger volume of the sample into the capillary [12]. Although the injection of organic solvent causes a disturbance of current [13], there was no troublesome effect on the migration times or electropherograms of SA, aspirin and the other chemicals examined.

Fig. 1C is a representative electropherogram of the serum of a volunteer who had taken 990 mg of aspirin 3 h earlier. The SA peak was clearly found, but aspirin, GA, and SU were not detected, probably because of their low levels in the serum. Fig. 1A is an electropherogram of drug free human serum pretreated with acetonitrile including I.S. This indicates that there were no peaks interfering with the objective peaks in the serum.

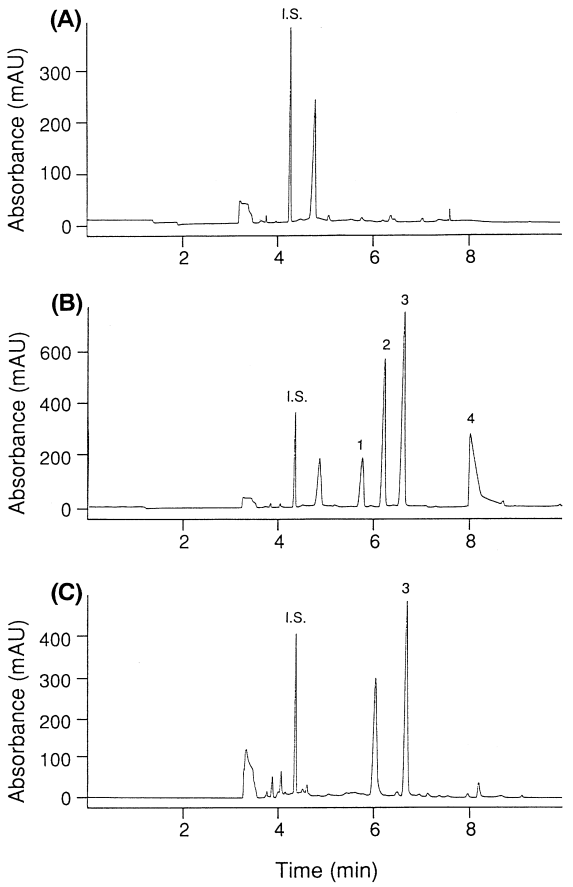

Fig. 1. Electropherograms obtained from the extracts pretreated by acetonitrile including 3-isobutyl-1-methylxanthine (50 μg/ml; as internal standard) of (A) drug free human serum, (B) human serum spiked with standard mixtures of aspirin (125 μg/ml; 1), gentisic acid (125 μg/ml; 2), salicylic acid (125 μg/ml; 3) and salicyluric acid (125 μg/ml; 4), and (C) serum of volunteer who took aspirin 990 mg.

The qualitative characterization of the peak of each substance was carried out using diode array detection. Fig. 2 shows the UV spectra between 190 and 400 nm for the standard I.S., aspirin, GA, SA and SU corresponding to the peaks in the electropherogram shown in Fig. 1B. The spectral comparison of the peaks from standard chemicals showed

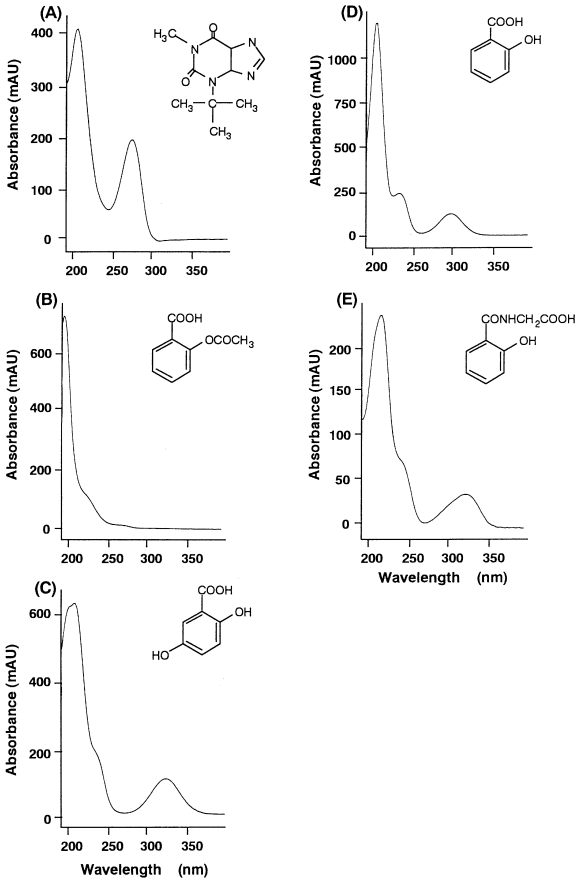

Fig. 2. Normalized spectra of (A) 3-isobutyl-1-methylxanthine (as internal standard), (B) aspirin, (C) gentisic acid, (D) salicylic acid, and (E) salicyluric acid obtained as slices from data in Fig. 1B, and their chemical structures.

that the peaks of the volunteer's serum were from SA and I.S.

3.2. Stability of analyte

The aspirin spiked in the serum was hydrolyzed to SA, and the half-life of aspirin was about 2 h at 37°C incubation (Fig. 3). The addition of 0.5 m*M* DFP to serum did not completely inhibit the hydrolyzation of aspirin. Eserine sulfate at 10 m*M* prolonged the half-life of aspirin to 8 h, but about 40% of the aspirin was hydrolyzed 3 h after the start of incubation. One-hundred μg/ml aspirin with and without 10 m*M* eserine sulfate were not detected after 24 h incubation at 37°C. Furthermore, although potassium

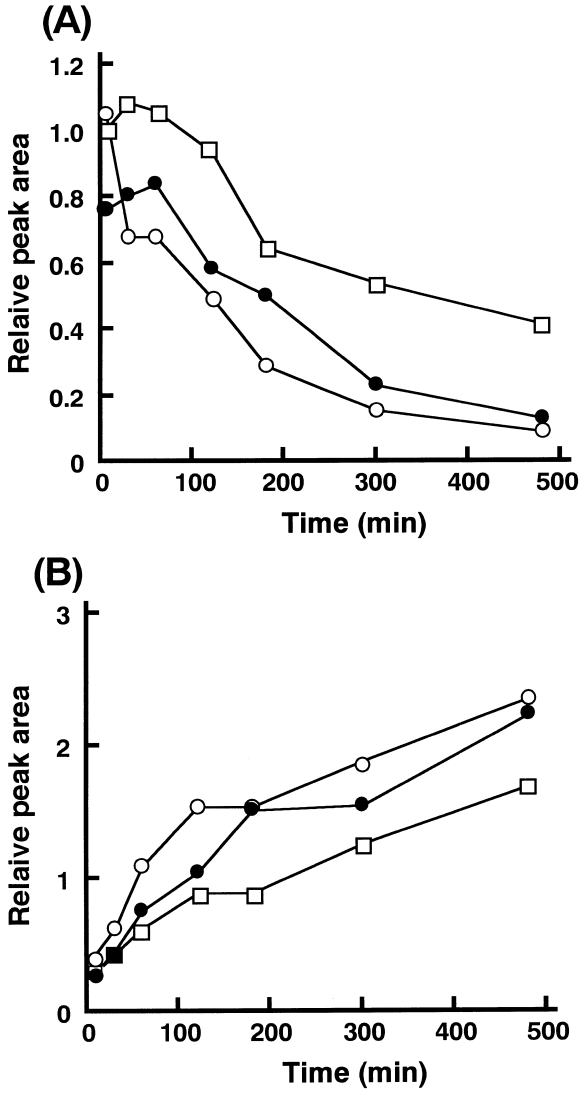

Fig. 3. The effect of incubation time at 37°C on (A) the hydrolysis of aspirin and (B) the formation of salicylic acid in serum without inhibitor (○), with 0.5 m*M* diisopropyl fluorophosphate (●) and with 10 m*M* eserine sulfate (□).

fluoride is widely used as the inhibitor of aspirin hydrolyzation, 0.5% of this reagent in serum disturbed the electropherogram (data not shown) [14]. Therefore, an assay for aspirin should be done immediately after sampling. In this study, however, the serum was incubated overnight after sampling, because (1) the addition of esterase-inhibitor to blood just after sampling interferes with the choline-

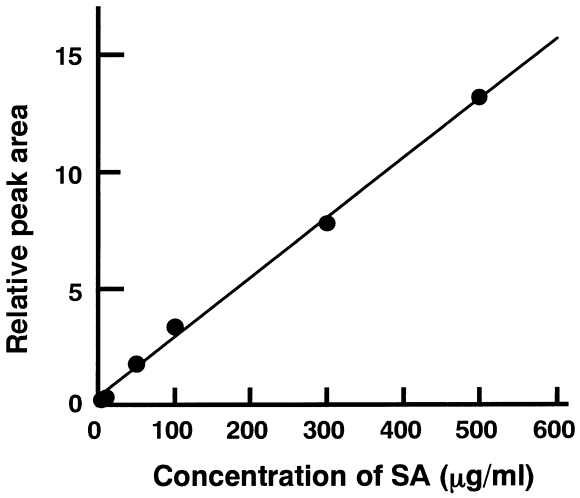

Fig. 4. Calibration curve for salicylic acid ($y=0.318+2.562\cdot 10^{-2}x$, $r=0.998$).

esterase activity which is often examined clinically, (2) patients' samples are not always brought to the assay department immediately after blood collection, (3) hydrolyzation in serum cannot be stopped completely by the addition of esterase- inhibitor even if the serum is stored at −20°C [15,16] and (4) the aspirin serum levels of patients at the trough level just before administration are very low [6].

The absolute analytical recoveries of SA spiked in serum were calculated by the assay in the calibration of SA spiked in deionized water. The recoveries of SA were 90.4% ($n=3$, 5 μg/ml), 111.3% ($n=3$, 10 μg/ml), 108.0% ($n=3$, 50 μg/ml), 92.1% ($n=3$, 100 μg/ml), 97.4% ($n=3$, 300 μg/ml) and 97.6% ($n=3$, 500 μg/ml).

3.3. Calibration

The calibration curve of SA in the range from 5 to 500 μg/ml is demonstrated in Fig. 4. The correlation coefficient (r) between the SA concentration and relative peak areas was 0.997–0.998 ($n=5$). This result showed the usefulness of the present CZE method following acetonitrile deproteinization in the assay of SA from low to high serum levels. The detection limit of SA (signal-to-noise ratio of 3) was 1.0 μg/ml, suggesting that this method is more sensitive than the FPIA method.

The intra- and inter-day reproducibility of repeated SA assays are shown in Table 1. In the intra-day assay, the coefficients of variation of the migration time and relative peak area were 0.15–0.49 and 0.24–2.66% in all concentrations, respectively. In the inter-day assay, these values were 0.83–1.44 and 4.39–8.73%, respectively. These results suggest that separation and quantification with this CZE system are reproducible for the measurement of SA levels in human serum.

Table 1
Reproducibility of migration time and relative peak area of salicylic acid

Concentration (μg/ml)	Migration time (min)	Relative peak area
Intra-day assay		
5	6.553±0.018 (0.27)	0.218±0.003 (1.17)
10	6.549±0.019 (0.28)	0.342±0.009 (2.66)
50	6.550±0.027 (0.40)	1.778±0.012 (0.66)
100	6.551±0.032 (0.49)	3.385±0.016 (0.46)
300	6.588±0.010 (0.15)	7.580±0.018 (0.24)
500	6.641±0.023 (0.35)	12.302±0.044 (0.36)
Inter-day assay		
5	6.557±0.093 (1.42)	0.201±0.018 (8.73)
10	6.584±0.095 (1.44)	0.398±0.029 (7.32)
50	6.572±0.065 (0.98)	1.694±0.099 (5.84)
100	6.577±0.055 (0.83)	3.387±0.149 (4.39)
300	6.620±0.067 (1.01)	7.828±0.496 (6.34)
500	6.664±0.080 (1.20)	12.747±0.594 (4.66)

Values are mean±S.D. ($n=5$). The values in parentheses are coefficients of variation (%).

3.4. CZE and FPIA

We determined the concentrations of SA in volunteers' sera by CZE and FPIA methods. As shown in Fig. 5A, there was a good correlation between the SA concentrations determined by two methods (r= 0.973, n=12), although the levels determined by CZE were slightly higher than those by FPIA (y intercept=6.8 μg/ml). The bias method was used to examined the serum levels of each SA analysis; i.e., the difference versus mean of each pair of data were plotted [17], and all samples were found to be within the range defined by the mean of differences ±2 S.D. When the TDx calibrator for SA was deprotenized with acetonitrile and assayed by the CZE method, a peak which seemed to be the other metabolite of SA was found. The FPIA for SA has been reported to show a high cross-reactivity to some kinds of SA metabolites [4]. This might be one of the reasons for the present difference between the values determined by CZE and FPIA.

When the CZE method was applied to a juvenile rheumatoid arthritis patient, the SA level 2 h after the patient was administered 960 mg aspirin was 38.7 μg/ml, and it decreased to an undetectable level at 12 h. This value was comparatively lower than that determined in a volunteer administrated 990 mg aspirin (Fig. 5A). This may be due to an autoinduction of SA metabolism on chronic administration [2]. In five patients taking low doses of aspirin continuously, the trough levels of serum SA were under detection limit.

3.5. Characteristics of CZE

Based on these results, the CZE assay appears to be useful for monitoring SA in human serum. This method is highly sensitive, with a detection limit in the 10^{-6} g/ml range. Regarding aspirin and SA assays when lower doses of aspirin are used, further study is necessary to determine the optimal conditions for the complete inhibition of aspirin hydrolyzation, electrophoresis conditions, which will not disturb the electropherogram even with the use of such an inhibitor, and more sensitive detection techniques. This CZE method is a viable alternative to the FPIA method, and more attractive than HPLC in light of the high running cost, system maintenance and use of organic solvent involved in the use of HPLC.

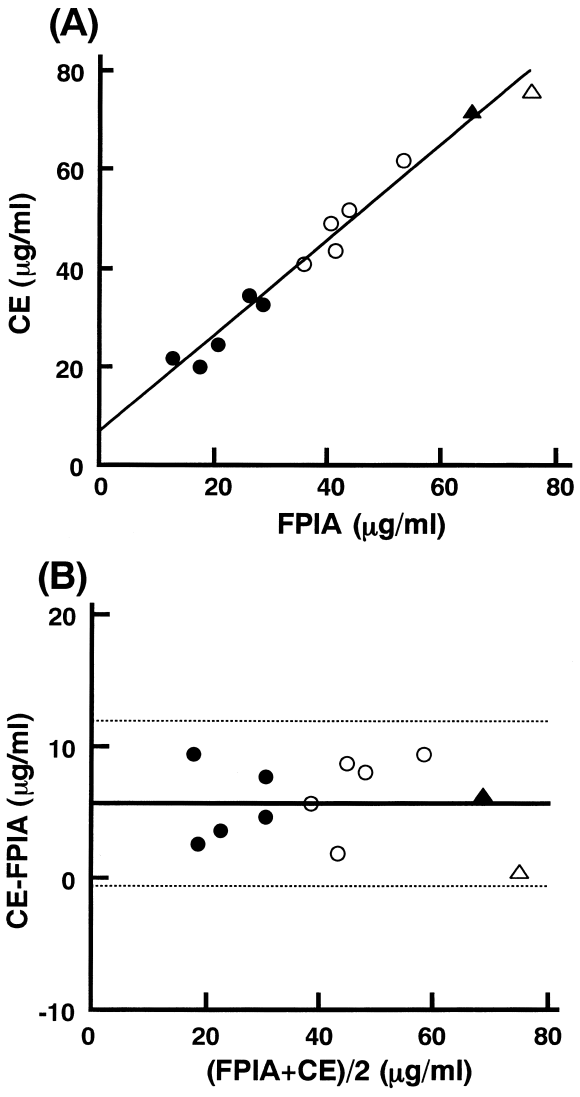

Fig. 5. Comparative salicylic acid levels in the 12 volunteers' sera determined by CZE and FPIA. The samples were obtained 2 h (○) and 5 h (●) after administration of 660 mg aspirin, and 2 h (△) and 3 h (▲) after administration of 990 mg aspirin. (A) CZE versus FPIA by linear regression analysis. (B) Bias method analysis defined by the difference versus mean of comparative drug levels. The solid line in panel A is a regression line. In panel B, the solid line represents the mean of differences (5.65 μg/ml) and broken lines represent this mean ±2 S.D. (+11.89/−0.59 μg/ml).

4. Conclusions

The CZE method with acetonitrile deproteinization could provide a simple and sensitive monitoring method for serum SA as an alternative to FPIA analysis, in patients who have consumed anti-inflammatory therapeutic or toxic doses of aspirin.

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