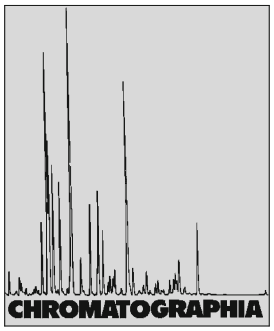
An In Vitro Study of the Interaction of Copper(II) Salicylates with Genomic DNA Using Gel Electrophoresis and Gel Permeation Chromatography



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Abstract

This papers describes the in vitro interaction of copper(II) acetylsalicylate and copper(II) salicylate with genomic DNA isolated from human blood. The two drug substances were found to bind to DNA after incubation with whole blood over night. Bonding was confirmed by detection of separated DNA electrophoresis bands for copper, copper(II) acetylsalicylate, copper(II) salicylate, acetylsalicylic acid and salicylic acid. Drug–DNA interactions were observed during electrophoresis in the form of fragmentation by formation of two bands when compared to controls. Gel permeation chromatography parameters also confirmed the occurrence of fragmentation. The use of gel permeation chromatography parameters as a measure of fragmentation of DNA is discussed. The fragmentation of genomic DNA after incubation with copper(II) acetylsalicylate and copper(II) salicylate suggested that these drug substances might be responsible for cytotoxicity in vivo.

Keywords

Gel eletrophoresis
Gel permeation chromatography
Drug–DNA interaction
Copper–DNA interaction
Copper-acetylsalicylate
Copper-salicylates

Introduction

Acetylsalicylic acid (ASA) is used for the treatment of rheumatoid arthritis for more than a century. The main adverse effect associated with ASA involves the formation of peptic ulcers [1, 2]. In an attempt to counter this adverse side effect, Sorenson [3] carried out pioneering work and reported that copper(II) acetylsalicylate (CAS) was several times more active than ASA and that anti-ulcer activity was also observed. The claimed enhanced anti-inflammatory activity and the anti-ulcer properties of CAS have been the subject of several research papers [4–6] and its potential as an ulcer-safe drug has been recognized. The compound is also currently in use for the treatment of rheumatoid arthritis [7–12]. In Pakistan, the commercially available preparation Nuhas, which contains CAS as the active ingredient, is used for the treatment of this condition for the last several years although it does not appear to be a registered drug.

Recently, we reported its pharmacokinetic study which supported the claim of enhanced activity [13]. In order to justify the use of copper(II) acetylsalicylate as a candidate drug it is a mandatory requirement to study its toxicity profile. When investigating the interaction between cupric chloride and genomic DNA it was found that DNA-bound copper mediated DNA base modifications and that non-bound copper mediated strand breakage [14]. The present work was carried to study the interaction between copper(II)

Table 1. Composition of various solutions

Solution	Composition	
TBE[a]	Trizma base	108.000 g
	Boric acid	55.000 g
	0.5 M EDTA	40 mL
	DI water	q. s. to make 1 L
		Dilute 10 mL of this to 100 mL with water
0.5 M EDTA[a]	EDTA	186.100 g
	NaOH	q. s. to dissolve EDTA
	DI water	q. s. to make 1 L
1 M Tris–HCl (pH = 8.0)[a]	Trizma base	121.100 g
	Conc HCl	q. s. to adjust pH to 7.4
	DI water	q. s. to make 1 L
Tris–EDTA[a]	1 M Tris–HCl	1 mL
	0.5 M EDTA	1 mL
	DI water	q. s. to make 100 mL
TNE[a]	1 M Tris–HCl	5 mL
	0.5 M EDTA	2 mL
	5 M NaCl	40 mL
	DI water	q. s. to make 500 mL
10% SDS	Sodium dodecyl sulphate	10.000 g
	DI water	q. s. to make 100 mL
PCI	Buffered phenol (Sigma, USA)	250 mL
	Chloroform	240 mL
	Iso-amyl alcohol	10 mL
Ethidium bromide	Ethidium bromide	500 mg
	DI water	10 mL

[a] The solution was autoclaved for 20 min at 120 °C

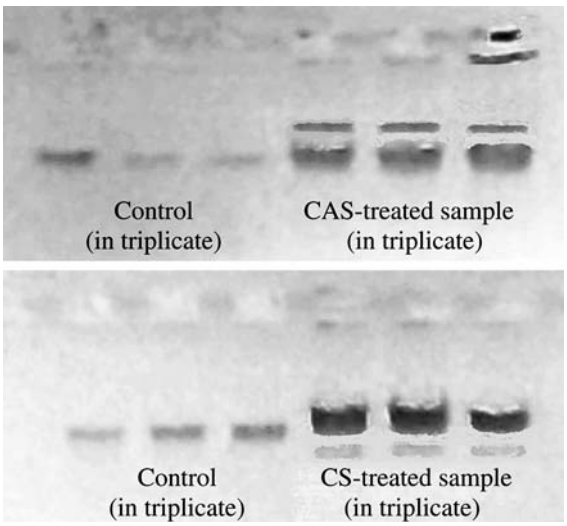

Fig. 1. Electrophoresis of control and treated DNA from experiment 4

acetylsalicylate and its metabolite, copper(II) salicylate (CS), with genomic DNA to obtain some basic information with regard to toxicity and efficacy of the candidate drug. Gel electrophoresis and gel permeation chromatography (GPC) were used to study this interaction.

Materials and Methods

Materials

Copper(II) acetylsalicylate [15] and copper(II) salicylate [16] were prepared in our laboratory by reported methods. Sodium chloride (Merck, Darmstadt, Germany), isoamyl alcohol (Merck), chloroform (Riedel-de Haen, Seelze, Germany), acetic acid 100% (Riedel de Haen), glycerol 87% (Merck), hydrochloric acid 37% (Merck), agarose (Bioline), boric acid (Merck), sodium hydroxide (Merck), proteinase-K (Sigma-Aldrich), ethanol (Merck), bromophenol (Merck), trizma base (Bioworld, USA), sodium dodecyl sulphate (Merck), EDTA (Merck), sodium acetate (Merck) and sodium citrate (Merck) were used as received.

Blood Samples

Heparinized human blood samples were collected from six volunteers (healthy males, age 22–27 years, weight 55–75 kg) and kept frozen at −20 °C for 2 days before use.

Isolation of DNA

Solutions used in this study were prepared from analytical grade reagents with their compositions as given in Table 1. DNA was isolated from both the control (blank blood) and treated (drug–DNA) samples. The frozen blood sample was thawed and a 500 μL aliquot was added to a Tris–EDTA solution (1,000 μL). The contents were mixed and centrifuged at 6,000 rpm for 6 min. The supernatant was discarded and the formed pellet was broken by gentle tapping. A Tris–EDTA solution (1,000 μL) was added again and the above procedure was repeated until the pellet became light pink. The supernatant was discarded leaving about 0.5 mL behind. TNE (300 μL), 10% SDS (15 μL) and proteinase-K (5 μL) were then added followed by an overnight incubation at 37 °C with shaking in order to ensure complete digestion of the pellet. The tubes were placed on ice and 6 M NaCl (50 μL) was added followed by addition of the PCI solution (20 μL). Each tube was shaken vigorously and placed on ice again for 5–10 min. This was centrifuged at 6,000 rpm for 6 min to give three layers. The upper layer contained DNA, the middle layer contained plasma membrane proteins and the lower most layer contained fats. The upper layer was carefully removed and added to pure ethanol, shaken well and centrifuged at 6,000 rpm

for 6 min. The supernatant was removed and the DNA pellet was washed with 70% ethanol and air-dried at 37 °C.

To the isolated DNA Tris–EDTA solution (100 μL) was added and the tube was incubated for 30 min at 37 °C with shaking for dissolution. The tube was covered with Parafilm and placed in a water bath at 70 °C for 1 h to inactivate nucleases. After this time the solution was allowed to cool to room temperature and the final concentration was adjusted to 50 μg mL^{-1} with the Tris–EDTA solution using the UV absorbance ratio (A_{260}/A_{280}) method and stored at −20 °C. This was labeled as the stock solution.

Drug–DNA Interaction Study

About 5 mg of the drug (CAS or CS) was added to a blood sample (20 mL), mixed gently and incubated over night at 37 °C. DNA isolation was carried out as described above and subjected to electrophoresis and GPC analysis in triplicate.

Gel Electrophoresis

Agarose gel (0.8%) was prepared in TBE followed by addition of ethidium bromide (1 μL). The solution was allowed to cool, poured into a casting tray containing two combs and allowed to solidify at room temperature. The combs were removed and the gel, having 32 wells, was inserted horizontally in the electrophoresis chamber containing the TBE solution (300 mL). The apparatus was connected to the 110 V power supply and current was allowed to pass for 30 min. Electrophoresis was performed using DNA solutions 1–4 (treated and control) that were prepared as follows. Solution 1: DNA stock solution (1 μL), dye solution (2 μL) and distilled water (9 μL) were mixed. Solution 2 consisted of DNA stock solution (2 μL), dye solution (2 μL) and distilled water (8 μL), solution 3 was prepared from DNA stock solution (3 μL), dye solution (2 μL) and distilled water (7 μL) whereas solution 4 was prepared by mixing DNA stock solution (6 μL), dye solution (2 μL) and distilled water (4 μL), respectively. Visualization of DNA was by staining with ethidium bromide and ultraviolet radiation. Photographs were taken after completion of the electrophoresis.

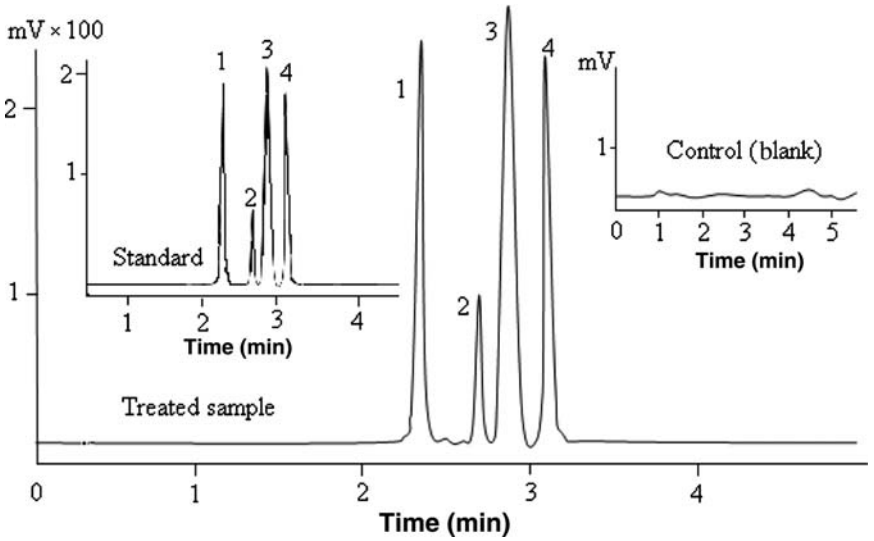

Fig. 2. Typical chromatograms showing separation of **1** = CAS, **2** = CS, **3** = ASA, and **4** = SA in standard, control (blank) and treated sample (DNA band in the electrophoretogram)

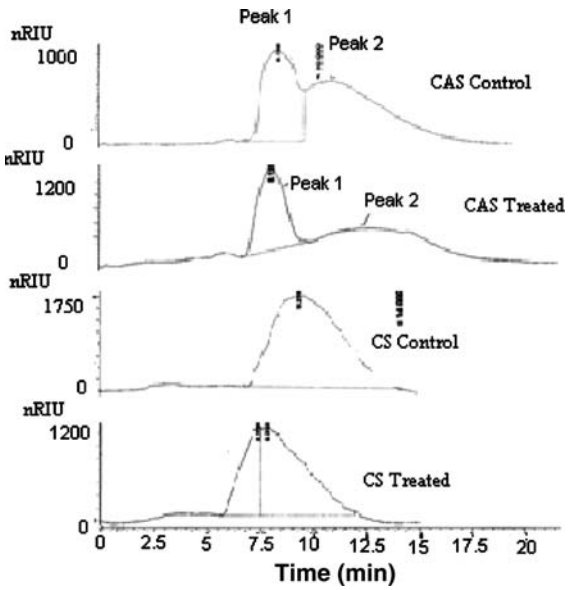

Fig. 3. Gel permeation chromatograms of control and treated DNA samples

Gel Permeation Chromatography

The DNA solutions prepared from treated and control samples were used for GPC analysis. An Agilent 1200 Series GPC-SEC Analysis System equipped with a quaternary pump, a refractive index detector and a PL Aquagel-OH 50A (8 μm) mixed column (7.5 × 300 mm) was used. The chromatographic conditions were as follows. Column temperature 37 °C, mobile phase Tris–EDTA, flow rate 1.00 mL min^{-1}, injection volume 20 μL and run time 20 min, respectively.

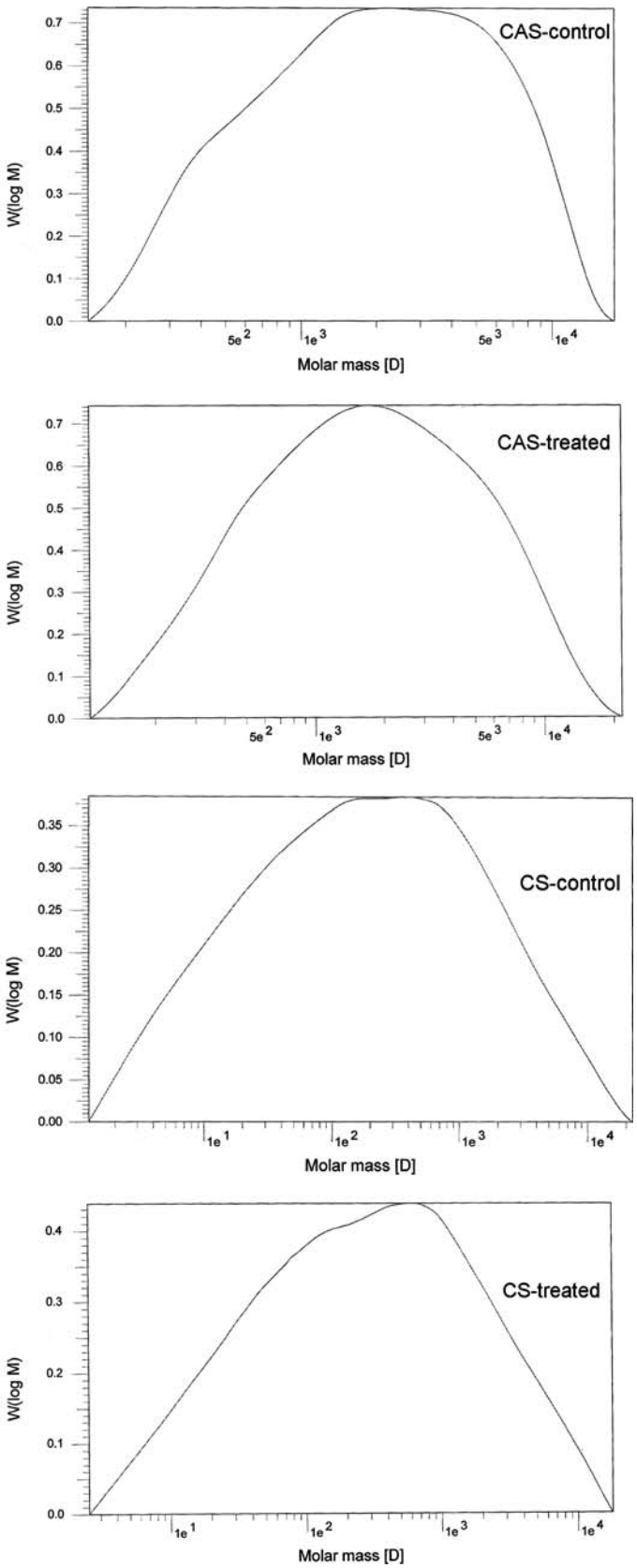

Fig. 4. Molar-mass-distribution curves of control and treated DNA samples

Results and Discussion

Electrophoresis

The isolated DNA aliquots obtained from control and treated blood samples were subjected to electrophoresis using dilutions 1–4 that derived from the stock solution. It was found that solution 3 produced well-defined bands thus the subsequent drug–DNA interaction experiments were studied using this dilution. A typical photograph taken from the resulting electrophoresis is shown in Fig. 1.

The DNA bands were removed and eluted with a 30% (*v*/*v*) nitric acid (for copper analysis) and methanol–acetic acid (20:01) mixture (for CAS and related substances). Copper analysis was carried out by flame-less atomic absorption spectrometry and by HPLC for CAS, CS, ASA or SA [10]. Representative chromatograms of standards and samples are shown in Fig. 2.

The results indicated the presence of the entire drug components attached to the bands derived from the treated sample whereas these were not detectable in the control sample. This confirmed that the bonding of CAS and CS with genomic DNA. The bonding may have occurred through a ligand-exchange process since CAS had four acetylsalicylate ions attached to two copper ions. It was therefore reasonable to expect that one or two of the ligands can exchange with other ligands available in the DNA structure found in the control-based single band. In case of treated samples two bands were observed which indicated bonding and fragmentation of DNA after interaction with the drugs under investigation.

Gel Permeation Chromatography

The GPC results are presented in the form of representative chromatograms (Fig. 3), molar-mass-distribution curves (Fig. 4) and in tabular form (Table 2).

Determination of GPC parameters included number average molecular mass (M_n), weight average molecular mass (M_w), atomic number average

Table 2. GPC parameters of control and treated DNA samples

Parameters	CAS		CS	
	Control	Treated	Control	Treated
M_n, g mol^{-1}	1,115.2	931.8	283.1	554.1
M_w, g mol^{-1}	3,065.2	2,792.8	954.2	1,090.1
M_z, g mol^{-1}	5,829.8	5,947.0	5,298.3	4,836.8
D	2.75	3.00	3.37	1.97
V_p, mL	7.98	8.10	8.76	8.55
M_p, g mol^{-1}	2,253.0	1,716.2	393.96	626.56
A	1,184.9	1,529.7	3,715.7	2,198.9

M_n Number average molecular mass, M_w weight average molecular mass, M_z atomic number average molecular mass, D polydispersity index, M_w/M_n, V_p volume at peak maximum, M_p molecular mass at peak maximum, A peak area

molecular mass (M_z), polydispersity index, M_w/M_n(D), volume at peak maximum (V_p), molecular mass at peak maximum (M_p), and peak area (A). A comparison of the GP chromatograms of the control and the treated DNA samples (Fig. 3) indicated significant differences in peak shapes. In case of CAS the ratio of peaks 1 and 2 of the control was about 1.5 times that of the treated sample and in case of CS the single broad peak of the control appeared to split into two after treatment. Similarly, the shapes of molar-mass-distribution curves of the control and the treated samples were also significantly different from each other (Fig. 4).

It was also found that in case of CS the polydispersity index of the control was about 1.7 times higher than that of the treated sample and these values were comparable with CAS.

Conclusions

The occurrence of distinctive interactions between copper(II) acetylsalicylate and copper(II) salicylate with genomic DNA was determined using gel electrophoresis and GPC parameters. Copper salicylates were found to bind with DNA leading to fragmentation. Whether this phenomenon might be responsible for anti-tumor activity of copper salicylates as suggested previously [17] remains to be investigated in more detail.

Acknowledgment

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