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Spectroscopic studies of the interaction of aspirin and its important metabolite, salicylate ion, with DNA, A·T and G·C rich sequences

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ABSTRACT

Among different biological effects of acetylsalicylic acid (ASA), its anticancer property is controversial. Since ASA hydrolyzes rapidly to salicylic acid (SA), especially in the blood, interaction of both ASA and SA (as the small molecules) with ctDNA, oligo(dA·dT)$_{15}$ and oligo(dG·dC)$_{15}$, as a possible mechanism of their action, is investigated here. The results show that the rate of ASA hydrolysis in the absence and presence of ctDNA is similar. The spectrophotometric results indicate that both ASA and SA cooperatively bind to ctDNA. The binding constants (K) are $(1.7 \pm 0.7) \times 10^3$ M^{-1} and $(6.7 \pm 0.2) \times 10^3$ M^{-1} for ASA and SA, respectively. Both ligands quench the fluorescence emission of ethidium bromide (Et)–ctDNA complex. The Scatchard plots indicate the non-displacement based quenching (non-intercalative binding). The circular dichroism (CD) spectra of ASA– or SA–ctDsNA complexes show the minor distortion of ctDNA structure, with no characteristic peaks for intercalation of ligands. T_m of ctDNA is decreased up to 3 °C upon ASA binding. The CD results also indicate more distortions on oligo(dG·dC)$_{15}$ structure due to the binding of both ASA and SA in comparison with oligo(dA·dT)$_{15}$. All data indicate the more affinity for SA binding with DNA minor groove in comparison with ASA which has more hydrophobic character.

1. Introduction

Using the powder extracted from the bark of willow to treat pain and reduce fever can be traced back to the fifth century BC. In 1829, salicin (the parent of the salicylate) was isolated from willow bark. Sodium salicylate was developed along with salicylic acid (SA) in 1875 as a pain reliever. In 1897 *Felix Hoffman* made aspirin (acetylsalicylic acid or ASA, Fig. 1), with reduced effect of irritating the stomach. Today aspirin is used not only as a painkiller, but also as an inhibitor of prostaglandin release. It also affects different pathways and has been known for various pharmacological properties, including: increasing blood vessel elasticity, acting as an anti-inflammatory drug, inhibition of platelet aggregation and blood clotting [1–4]. Furthermore, its beneficial effects on treatment of cataract [5], sickle cell [6] and type I diabetes mellitus [7] were reported.

The anticancer effect of aspirin in some cancerous cell lines [8–10], through induction of apoptosis [11–16], inhibition of DNA [8,16] or protein synthesis [8], inhibition of proteasome function [17] and at higher doses, the inhibition of angiogenesis [18] have been reported. However, the controversial results about its benefit on cancer treatment or prevention in human and animal model of cancer were reviewed elsewhere [19,20]. In addition, the reduction in neuronal function recovery [21] and neurotic outgrowth by aspirin [22] were shown. Aspirin also activates some carcinogens such as *N*-hydroxyarylamines to produce carcinogen-DNA adducts [23–24].

Aspirin hydrolyzes spontaneously to salicylic acid through the intramolecular general base catalysis by a carboxylate group [25]. The kinetic parameters for ASA hydrolysis were reported in different media. The half-life of aspirin in rat blood at 37 °C is about 0.21 h (12.6 min) and it is hydrolyzed rapidly by arylesterases [26].

In 1968, it was reported that aspirin at therapeutic concentrations, acetylates a variety of body constituents, including plasma proteins such as albumin, IgG, IgA, transferrin [27]. Then, acetylation of endoperoxide synthesis (in cylooxygenase, Cox-1 and Cox-2) [28–31], thus inhibition of prostaglandines biosynthesis [32] was shown. Acetylating effect of ASA on some other proteins were reported in the next years [5,6,33,34].

On the other hand, the effect of salicylate ions, as the most important metabolite of ASA, on the inhibition of palmitate oxidation [35], growth of tumor cell lines [8,36] and angiogenesis in endothelial cells with the same potency as aspirin, through Cox-independent mechanism [18] were shown.

According to the above mentioned results, it seems that acetylation is not the only mechanism of aspirin action and salicylate ions also play an important role. Since, the exact mechanism of ASA action and the molecular target of both ASA and SA are not clear yet,

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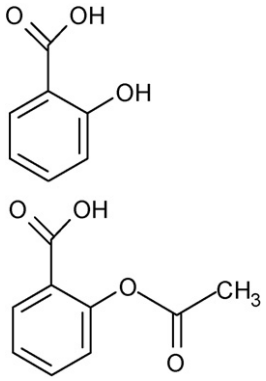

Fig. 1. The structural formula of SA (up) and ASA (down).

we attempt to investigate the interaction of these compounds with DNA and the preference of their binding to A·T or G·C sequences (oligo(dA·dT)$_{15}$ or oligo(dG·dC)$_{15}$), as a possible mechanism for some of their actions.

2. Experimental

2.1. Materials

Calf thymus DNA (ctDNA) was extracted by the method explained previously [37]. Fiftheen bases olignucleotides (dA, dT, dG and dC) were purchased from Cinagen. Co., Iran, Aspirin and salicylic acid were obtained from Sigma, tris was from Merck, and all of the other materials used were of analytical grade.

The experiments were carried out at pH 7.4; using 0.05 M tris buffer, at room temperature in the absence and presence of 1 M NaCl.

The ctDNA concentrations were determined using an extinction coefficient of 6600 M^{-1} cm^{-1} at 260 nm and expressed in terms of base molarity (Mb) or milli molar base (mMb) [38].

2.2. Ultraviolet spectrophotometry

The spectrophotometric measurements were made with Shimadzu Model-3100 Double-beam Spectrophotometer. Since, both aspirin and DNA show the absorption maximum at 260 nm, differential spectroscopy was selected as a method of choice. By this method a small change in the absorption is detectable. Thus, two sets of experiments (a and b) were designed as follows:

(a) Two cuvettes (sample and blank) containing 2.5 ml of ctDNA solutions, with the desired concentration, were titrated by ligand (ASA/SA) and buffer (10 μl in each injection), respectively. The absorbency (A_1) at each wavelength is related to the both free and bound ligand.
(b) The similar set of titration was done, using the cuvettes containing 2.5 ml of buffer only. The absorbency (A_2) at each wavelength is related to the free ligand.
(c) By assumption that the absorbency of the free ligand is similar in both sets of experiments, absorbance of the bound ligand (ctDNA–ligand complex) was obtained as: $\Delta A = A1 - A2$.

All calculations performed by the data processing mode of the spectrophotometer's software.

2.3. Spectrofluorometry

The Hitachi MPF-4 spectrofluorimeter, which was computerized in our lab, was used. The scan speed was 60 nm/min, both slits were 10 nm, λ_{ex} = 525 nm and λ_{em} = 584 nm and no filter used. The procedures for fluorometric studies of ctDNA–ethidium bromide (Et) complex in the absence and presence of ASA or SA have been performed according to the previously reported method [39].

2.4. Circular dichroism (CD)

CD measurements were made on a Jasco Model J-810 CD recorder at 25 °C. Data reported as molar ellipticity, [θ] (deg cm^2 $dmol^{-1}$), based on the average weight of nucleotide was equal to 330. The molar ellipticity for ctDNA was determined as $[\theta]_\lambda = (\theta \times 330)/cl$, where c is the ctDNA concentration in mg/ml, l is the light path length in centimeter, and θ is the measured ellipticity in degrees at a wavelength of λ. The molar ellipticity for oligonucleotides was determined by using their molecular weight.

3. Results and discussion

3.1. Spectrophotometry

Because of the rapid hydrolysis of aspirin in solutions, the kinetic of ASA hydrolysis in the buffer condition was first investigated. Some spectra at different concentrations of aspirin and at different days up to a week were plotted. Three different peaks were observed at 208 nm, 230 nm and 295.5 nm, as well as two isosbestic points at 245 nm and 268 nm in the spectra belong to each concentration at different day (Fig. 2A). The same set of spectra was plotted in the presence of ctDNA (Fig. 2B).

Overally, the decrement at 260 nm (valley production) and the increment in the peak at 295.5 nm are indicative of aspirin hydrolysis and salicylate production as a function of time [40]. The isosbestic point in the closed system indicates the conversion of the reactants to the products with a constant ratio and it is due to the linear changes of the different components in the reaction [41]. In other words, in this reaction aspirin has been converted directly to salicylic acid.

The kinetic parameters, including half-life ($t_{1/2}$) and *pseudo*-first order rate constant (k) for ASA hydrolysis calculated according to the incremental changes in absorbency at 295.5 nm versus the time at different concentrations of ASA in the absence (Fig. 2C) and presence of ctDNA (Fig. 2D) based on the Eqs. (1) and (2) [41]:

$$v = k[\mathrm{ASA}] \quad (1)$$

$$t_{1/2} = 0.693/k \quad (2)$$

By comparison of the UV-spectra of ASA, up to a week, in the absence and presence of ctDNA (Fig. 2A and B), some changes in the peaks are observed. They are including: the decrease in the peaks at 260 nm and 208 nm accompanying with a red shift at 208 nm. These changes indicate the ctDNA–ASA interaction. However, there are no significant changes at 295 nm in the presence or absence of ctDNA (Table 1) that indicate ctDNA has no effect on the rate of ASA hydrolysis.

Our experiments also indicate that if aspirin is prepared daily, it remains stable during the course of the reaction with almost no significant amounts of salicylate formation.

Table 1
Pseudo-first order rate constant and the half-life of aspirin hydrolysis in tris buffer 0.05 M, pH 7.4 in the presence and absence of DNA.

Substance	k (h^{-1})	$t_{½}$ (h)
ASA–DNA	0.0188 ± 0.0016	36.99 ± 2.83
ASA	0.0195 ± 0.0023	35.79 ± 3.39

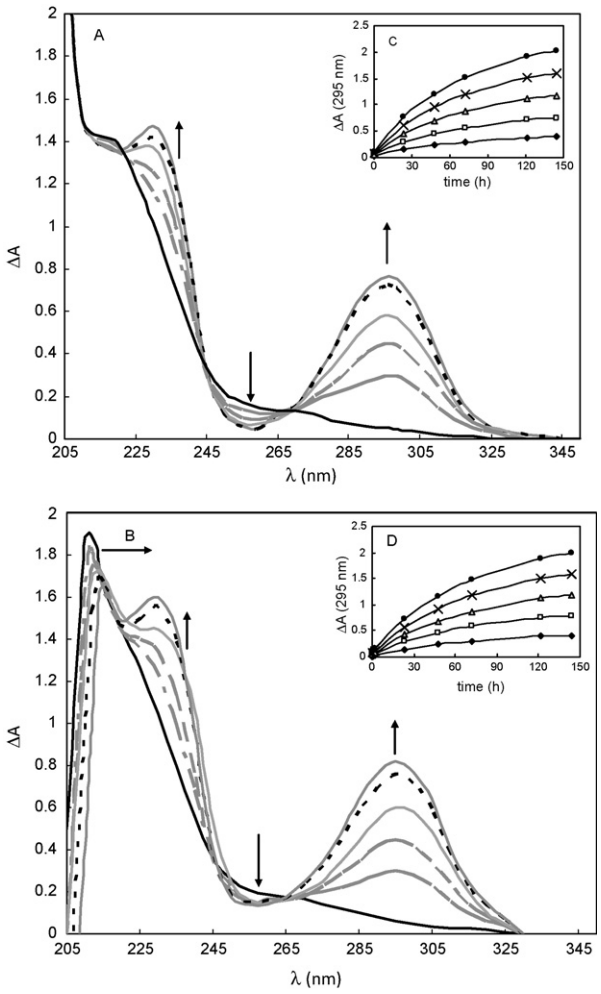

Fig. 2. Absorption spectroscopy. (A) Absorption spectra of ASA (0.125 mM) at 0 h, 24 h, 48 h, 72 h, 121 h and 144 h after preparation, at room temperature. (B) The same as before but in the presence of 25 μg/ml DNA. (C) Absorbance changes of different concentrations of ASA (0.125 mM (♦), 0.25 mM (□), 0.375 mM (△), 0.5 mM (×) and 0.625 mM (●) at 295.5 nm against time, which are used for determination of kinetic parameters. The plots for higher concentrations of ASA, 0.625–1.25 mM, are not shown. (D) The similar plots as (C) but in the presence of 0.147 mMb DNA. Arrows show the direction of changes in the spectra by time.

3.2. Differential spectroscopy

All molecules in this study (ctDNA, ASA and SA) have the absorbance peak at 260 nm, thus differential spectroscopy is used as the method of choice for investigating the interaction of ASA or SA with ctDNA. Thus as explained in the parts (a) and (b) of Section 2, interaction of different concentrations of ASA or SA with ctDNA were studied. Then, the changes in the absorbency (ΔA) at different wavelengths were calculated and plotted using the data processing software of the UV–vis Shimadzu Spectrophotometer Model 3100, as explained in part (c) of Section 2. The obtained data are plotted in the Fig. 3A–C. An increase in the absorbance at 260 nm after complex formation between ASA/SA with ctDNA is seen in Fig. 3A. Both plots have the sigmoidal shape. The changes (decrement) in the peak at 208 nm as well as the amount of the red shift ($\Delta\lambda$) of the peak at this wavelength, versus the ligand concentration are shown in Fig. 3B and C, respectively. All plots in Fig. 3 are indicative of ctDNA interactions with both ASA and SA. The more red shift observed at 208 nm in the presence of ASA is due to its more hydrophobic character in comparison with SA. The red shift due to more hydrophobic ligands was also reported elsewhere [42].

Binding parameters of ctDNA–ASA interaction are calculated according to the changes at 260 nm (Fig. 3A). One can fit the ΔA *vs.* [ligand] curve to the following equation:

$$\Delta A = \Delta A_{max}((K[\text{ligand}])^h/(1+(K[\text{ligand}])^h) \quad (3)$$

where h is the Hill coefficient, K is the equilibrium association constant, and ΔA_{max} is the maximum perturbation in the ctDNA absorbance. Analyzing the data of ASA binding is shown in Fig. 3A gives values of 1.8 ± 0.3 for h, $(1.7 \pm 0.7) \times 10^3\ M^{-1}$ for K, and 0.079 ± 0.033 for ΔA_{max}. A similar analysis for SA binding gives values of 7.2 ± 1.3 for h, $(6.7 \pm 0.2) \times 10^3\ M^{-1}$ for K, and 0.0042 ± 0.0002 for ΔA_{max}. The Hill coefficients indicate more cooperativity in the SA–ctDNA interaction in comparison with ASA binding to ctDNA, may be due to the steric hindranceof the additional acetyl group on ASA. The binding constants seem to be low, but they are comparable with that reported for other ligands such as

The cooperative binding constant (K_{Co}), which was first defined by Sano et al. [43], shows the binding strength regardless of the cooperativity parameter. It is obtained as:

$$K_{Co} = K_0 \times q \quad (4)$$

where k_0 is the intrinsic (or obtained) binding constant and q is the cooperative interaction parameter (or Hill coefficient). The average K_{Co} for SA and ASA interaction with ctDNA in this study is equal to $(2.5 \pm 0.3) \times 10^4\ M^{-1}$. This value is very similar to that recently obtained by us using electrochemical method to investigate the binding of ASA/SA with the immobilized ctDNA on the nanofiber polypyrrole electrode (unpublished data).

3.3. Spectrofluorimetry

Ethidium bromide (Et) fluorescence emission is increased upon interaction with ctDNA [44]. Both ASA and SA quench the fluorescence emission of ctDNA–Et complex with no effect on the emission of Et alone. The Scatchard analysis of the fluorescence data of ctDNA–Et complex in the absence and presence of different concentrations of ASA or SA are calculated as follows [39]:

$$I_{obs} = I_b + I_f \quad (5)$$

where the observed emission at any time of the experiment is I_{obs}, I_f and I_b are the fluorescence emissions of free and bound ethidium bromide. I_b and I_f are functions of the concentration of bound (c_b) and the remaining Et (c_f).

$$I_{obs} = k_b c_b + k_f(c_{total} - c_b) \quad (6)$$

The constants k_b and k_f are determined experimentally. c_{total} is controlled and c_b is determined by monitoring of I_{obs} using Eq. (7).

$$C_b = \frac{I_{obs} - k_f C_{total}}{k_b - k_f} \quad (7)$$

Once the concentration of the bound dye is known, the binding of the Et may be described in terms of the Scatchard equation:

$$\frac{\nu}{C_f} = nK - \nu K \quad (8)$$

where ν is the ratio of bound Et to ctDNA base pair; n is the maximum value of ν and K is the intrinsic binding constant of the Et.

Fig. 4A and B show the Scatchard plots of Et binding to ctDNA in the absence and presence of different concentrations of ASA or SA, respectively. These Figures indicate the changes in both the slope (K) and the intercept (n) of the Scatchard plot of Et–ctDNA complex upon the addition of ASA or SA (as a second ligand). The determined values of the K and n are shown in Table 2.

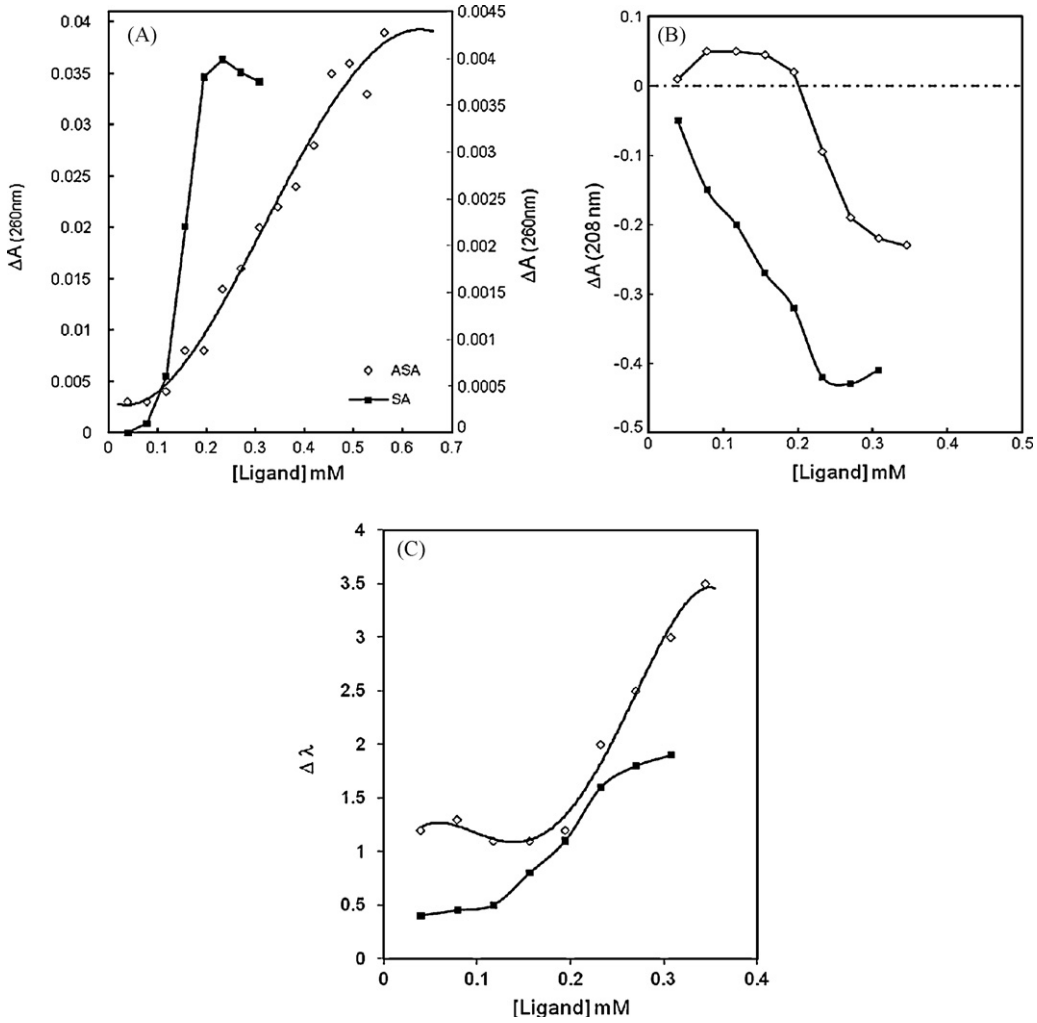

Fig. 3. The plots of the absorbency changes obtained by differential spectroscopy (as explained in Section 2): (A) at 260 nm for DNA–ASA (◊) or DNA–SA (■) complexes; (B) at 208 nm for DNA–ASA (◊) or DNA–SA (■) complexes; and (C) the amount of the red shift of the peak (Δλ) upon complex formation against the ligand concentration (similar marks as A & B were used). [DNA] = 25 μg/ml in tris buffer 0.05 M, NaCl 1 M, pH 7.4.

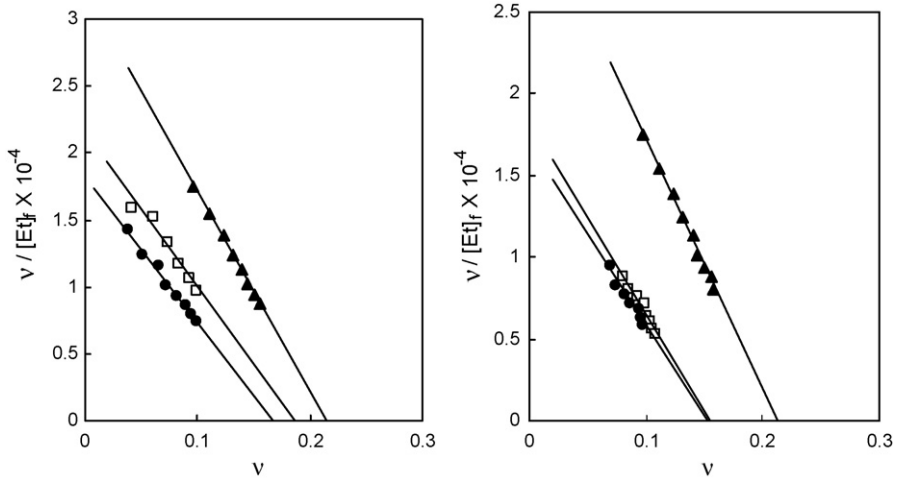

Fig. 4. The Scatchard plots for Et–DNA complexes in the absence and presence of ASA (left) or SA (right) in tris buffer 0.05 M, NaCl 1 M, pH 7.4. The Scatchard plot in left are shown as follows: Et–DNA complexes alone (▲), as well as 0.18 mM of ASA (□) and 0.37 mM of ASA (●). The plots in right are Et–DNA complexes alone (▲), 0.11 mM of SA (□) and 0.29 mM of SA (●). At concentrations more than 0.53 mM of the ligands, no more changes were observed.

Table 2
Binding parameters for Et–DNA complex in the absence or presence of different concentration of ASA or SA.

Ligand	mM	K (M^{-1})	n
Et (alone)		1.528×10^5	0.239
ASA + Et	0.18	1.485×10^5	0.171
	0.37	1.447×10^5	0.153
	0.53	1.437×10^5	0.146
SA + Et	0.11	1.146×10^5	0.180
	0.29	1.128×10^5	0.166
	0.53	1.103×10^5	0.153

Quenching of the emission of Et–ctDNA complex in the presence of various ligands was reported previously [39,45–50], and different Scatchard plots have been obtained for the observed behaviors [39,51,52]. According to the classification of Howe-Grant et al. [51] the changes in both K and n have named as *type B* or non-competitive behavior. The proposed mechanism for such behavior is energy transfer from excited ctDNA bases not only to an intercalated Et through direct contact but also to that bind in a minor groove [46,48–50,53]. The obtained results are also compatible with the results obtained by Neault et al. that indicated the binding of ASA to the backbone phosphate, as well as A–T and G–C base pairs of ctDNA through hydrogen bonding [54].

3.4. Circular dichroism (CD)

Fig. 5 shows the CD spectra of ctDNA in the absence and presence of Et, ASA or SA. As it is seen a significant peak at 308 nm is observed for ctDNA–Et complex (curve 4), which is a characteristic peak for the intercalation of Et between ctDNA base pairs [55,56]. In addition, the broad and positive bands at 275 nm as well as increasing in the negative band at 248 nm are other reasons for Et binding through intercalation [55,56]. The mentioned peaks are absent in the CD spectra of ASA– or SA–ctDNA complexes (curves 2 and 3). The small changes in the peaks at 275 nm and 248 nm are only due to the minor structural changes in the ctDNA upon ASA or SA bindings. These results confirm the non-intercalative mechanism of binding obtained by fluorescence data. However, the B-to A-ctDNA transition that was shown by Neault et al. [54] is not

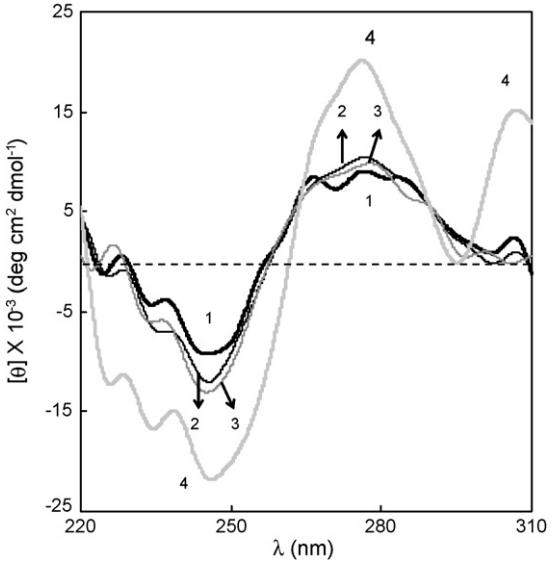

Fig. 5. The spectropolatimetry. The CD spectra of DNA (0.112 mMb) alone (curve 1); DNA–ASA (curve 2, 1.4 mM ASA); DNA–SA (curve 3, 1.4 mM SA and DNA–Et (curve 4, 8.4 μM Et).

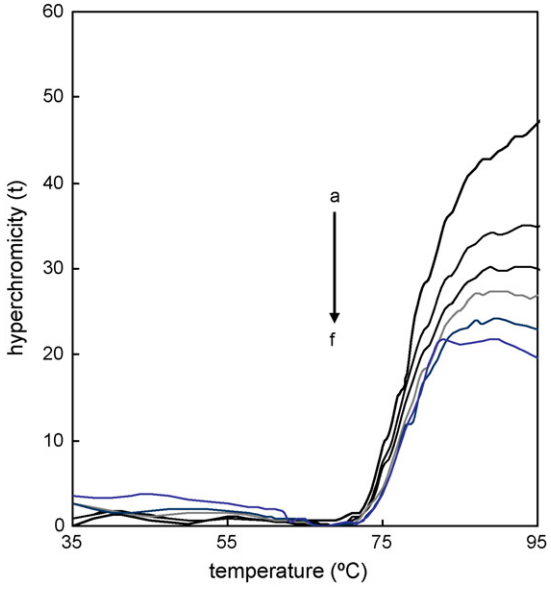

Fig. 6. The hyperchromicity of DNA due to the raising in the temperature in the absence (a) and prescence of different concentrations (0.125 mM, 0.25 mM, 0.5 mM, 0.75 mM, 0.875 mM, which were named b to f) of ASA.

confirmed in this study. It is important to note that they solved ASA in methanolic solution, which may affect the ctDNA conformation. In addition, the concentration of both ctDNA and ASA was very high at that study. Interaction of aspirin with RNA, through the G–C and A–U base pairs and the backbone PO_2 group, was also shown. Such interaction conserves the RNA structure in the A-conformation [57].

3.5. T_m determination

The hyperchromicity of ctDNA with increasing temperature in the absence or presence of different concentrations of ASA is also investigated (Fig. 6) and the T_m of ctDNA is calculated according to the method of Li using Eqs. (9) and (10) [58]. With 3 °C decrease in the T_m of ctDNA, it reached to 77 °C in the presence of ASA (compared to its initial value of 80 °C in the absence of ASA). As it is reported previously, reduction in the T_m is a characteristic of all known reagents that can form hydrogen bonds with nucleotides [59]. This result also confirms the non-intercalative mechanism of binding and induction of minor instability on ctDNA structure upon interaction with ASA [54].

$$h(t) = \frac{A(t) - A_0(t)}{A_0} \times 100 \quad (9)$$

$$\frac{\partial h(t)}{\partial t} = \frac{h(t+1) - (t-1)}{2} \quad (10)$$

where h is the hyperchromicity, A is the absorbance at each temperature and A_0 is the absorbance at beginning of the experiment.

3.6. Preferential binding

To study the preference of these ligands for different ctDNA sequences, their interaction with oligo(dA·dT)$_{15}$ and oligo(dG·dC)$_{15}$ are investigated by CD, Fig. 7.

A slight decrease in the ellipticity at 275 nm, accompanying with an increase in the negative peak at 248 nm is observed in the CD spectra of oligo(dA·dT)$_{15}$ after interactions with ASA or SA. The similar changes are observe in the CD spectra of oligo(dG·dC)$_{15}$,but at higher ligand/ctDNA ratio. These changes were leveled off after ligand/ctDNA molar ratio of about 2 in A·T sequence, but it began at higher molar ratio and continued to more than 5.5 in G·C sequence.

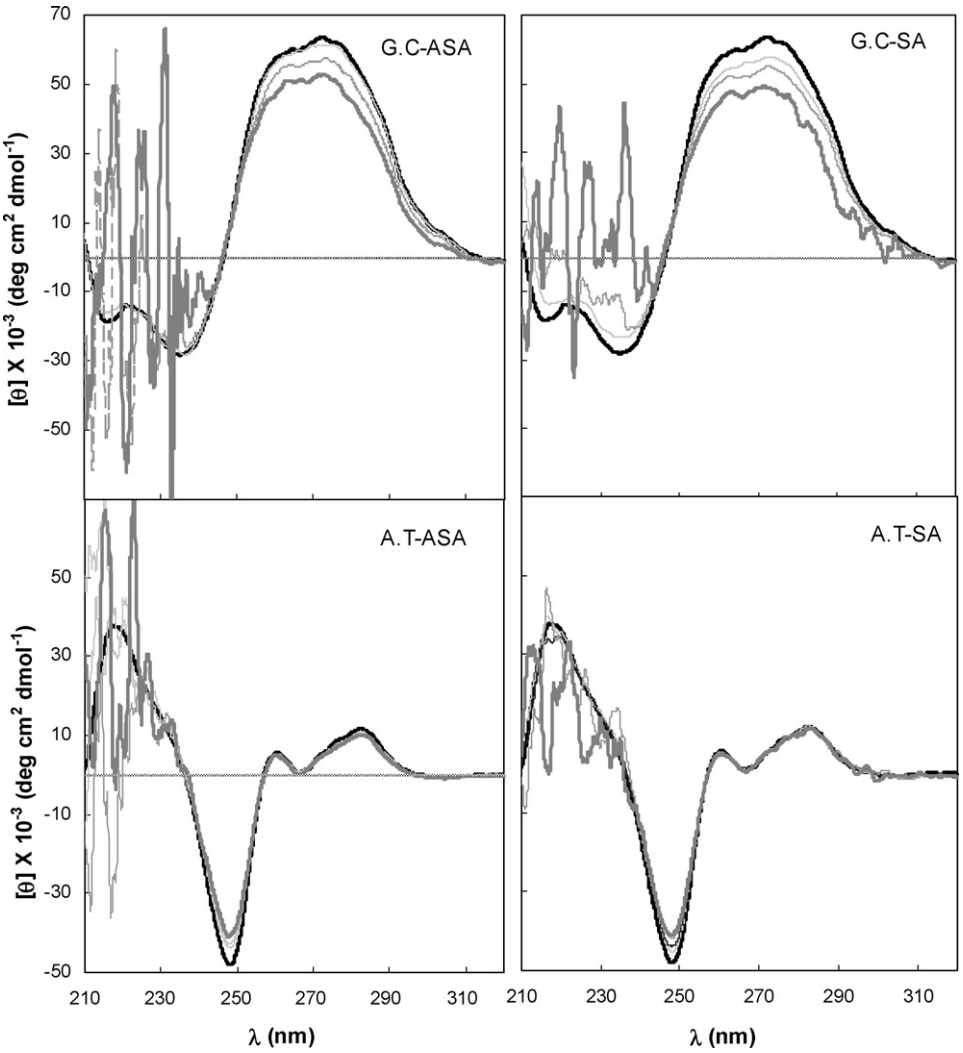

Fig. 7. The CD spectra of oligo(dG·dC)$_{15}$ titrated by ASA (G·C–ASA) and SA (G·C–ASA); and oligo(dA·dT)$_{15}$ titrated by ASA (A·T–ASA) and SA (A·T–SA). The oligonucleotide concentrations were 0.112 mMb and titration was done by ligands between 0 and 2.5 mM. In the case of G·C sequences, maximum changes were observed up to 2.13 mM of ASA and 2.28 mM of SA, but in the presence of A·T, after 1.47 mM of both ligands there were no more changes in the spectra >230 nm. In all cases by addition of more ligands the peaks below 230 nm destroyed completely (the plots not shown). In all figures the double stranded oligonuleotide is shown as black line, in the presence of ligand at the concentrations named above are shown as gray bold lines, and in the presence of lower ligand concentrations are shown as thin gray lines.

It indicates that interaction of ASA or SA with A·T sequences is happened at lower ligand concentration. These results are similar to that reported previously about interaction of ASA with ctDNA [54].

In addition, as it is seen in the figures (Fig. 7), there are some perturbations in the ellipiticity of each of the mentioned oligonucleotides at wavelengths below 240 nm. These changes are induced in the GC sequence at lower molar ratio of ligand/ctDNA than A·T sequence (0.92 and 1.13, respectively) and their intensities are more. The perturbations at lower wavelengths on the CD spectra may indicate some distortions on the hydrogen bonds [60].

Finally, based on the previous suggestion [21] and the results of this study, a more thorough investigation concerning the possible side effects of aspirin especially for its daily usage prescriptions [61], especially in children and during the pregnancy is suggested.

4. Conclusion

Both ASA and SA, as two known small molecules, interact with ctDNA through the groove binding. The ctDNA has no effect on ASA hydrolysis. Both ASA and SA induce little instability and minor distortion on ctDNA structure, which is confirmed by the CD spectrum and 3 °C decreases in the T_m of ctDNA. These are the characteristics of the non-intercalative ligands. By comparing these two ligands with each other, it shows that interaction of SA with ctDNA is more cooperative than ASA, but the latter induces more changes on ctDNA structure.

Both ligands interact with A·T oligonucleotide at lower concentrations in comparison with G·C sequence, and the changes induced by each of them (ASA or SA) in the A·T sequence are less than that induced on G·C sequence. It means that the observed changes in the G·C sequence is more serious and appeared at higher ligand/oligonucleotide molar ratio. The observed changes in the CD signals below 240 nm indicate that both ASA and SA affect the hydrogen bonding in the ctDNA structure, which is more in G·C oligonucleotide.

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