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Structure-activity study of thiazides by magnetic resonance methods (NQR, NMR, EPR) and DFT calculations

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Abstract

The paper presents a comprehensive analysis of the relationship between the electronic structure of thiazides and their biological activity. The compounds of interest were studied in solid state by the resonance methods nuclear quadrupole resonance (NQR), nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) and quantum chemistry (ab inito and DFT) methods. Detailed parallel analysis of the spectroscopic parameters such as quadrupole coupling constant (QCC) NQR chemical shift (δ), chemical shift anisotropy (CSA), asymmetry parameter (η), NMR and hyperfine coupling constant (A), EPR was performed and the electronic effects (polarisation and delocalisation) were revealed and compared. Biological activity of thiazides has been found to depend on many factors, but mainly on the physico-chemical properties whose assessment was possible on the basis of electron density determination in the molecules performed by experimental and theoretical methods.

Keywords: Structure-activity; Thiazides; NMR; NQR; EPR; DFT

1. Introduction

Sulfonamide derivatives of benzothiazidine, known as thiazides, are biologically active compounds of wide application in medical therapy [1–13]. Thiazides contain condensed rings with two nitrogen atoms and one sulphur atom in the heterocyclic ring and occur in two basic forms as hydrochlorothiazide (HCTZ, Fig. 1a) or chlorothiazide (CTZ, Fig. 1b) analogues. Only furosemide (FSE, Fig. 1c) being a derivative of sulfonamide belongs to thiazides and does not belong to any of the abovementioned groups. Thiazides are widely used as diuretics in the therapy of hypertension, anaphylactic shock, swelling, kidney diseases, mucoviscidosis, cancer and as drugs inhibiting osteoporosis [1–13].

This paper is a continuation of the cycle of works [14–20] devoted to analysis of the effect of the intra and intermolecular interactions on the electronic structure of thiazides in solid phase by the resonance methods: nuclear quadrupole resonance–NQR, nuclear magnetic resonance–NMR, and electron paramagnetic resonance–EPR. In the earlier works analysis of the spectroscopic parameters such as quadrupole coupling constant (QCC), NQR, chemical shift (δ), chemical shift anisotropy (CSA), asymmetry parameter (η), NMR and hyperfine coupling constant (A), EPR has been made, the possible structures and active sites have been proposed, the electronic effects (polarisation and delocalisation) estimated and the influence of irradiation on the electron density distribution in molecules established.

The above experimental methods provided information on the local distribution of electron density on the chlorine atoms (^{35}Cl-NQR), carbon atoms (solid state high resolution ^{13}C-NMR) or at the radical (EPR). Global analysis of electron density distribution in the whole molecule was made by the theoretical methods of *Density Functional Theory* (DFT). The criterion of their applicability was the correct reproduction of the molecule geometry and spectroscopic parameters obtained from experiment. In order to explain the electronic effects brought about by substitution at C(3) and relaxation processes in the molecular systems

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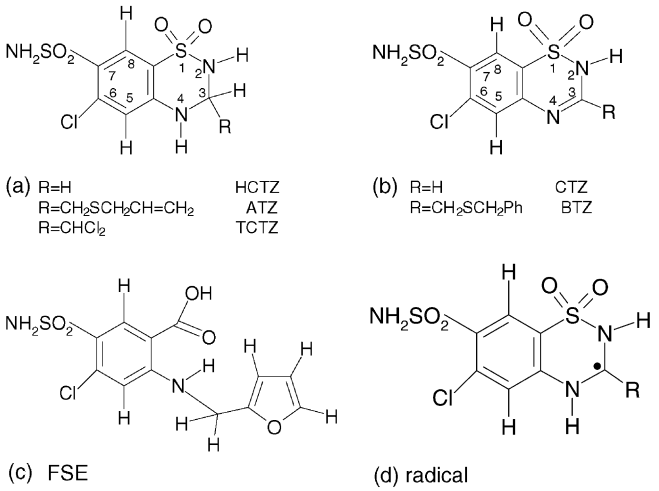

Fig. 1. Structural formulae of the thiazides studied.

studied, the DFT methods of *Natural Bond Orbitals* (NBO) and *Atom in Molecule* (AIM) were applied.

The aim of this paper is to find a correlation of the results hitherto obtained by experimental (resonance spectroscopy) and theoretical methods (DFT) for thiazides.

2. Experimental

Polycrystalline samples of hydrochlorothiazide (HCTZ), trichloromethiazide (TCTZ), althizide (ATZ) and chlorothiazide (CTZ) were purchased at Sigma–Aldrich and used without further purification.

The ^{35}Cl-NQR spectra of these compounds were taken at the liquid nitrogen and room temperatures. NQR signals assigned to Cl nuclei were weak ($S/N = 3$ after 1000 accumulations) and the resonant lines were very broad (even about 80 kHz), therefore, the classical Hahn sequence was applied. The optimised pulse length was about 13 μs. Due to the weak signals and broad resonance lines the accuracy of the frequency determination was not better than 10 kHz.

The ^{13}C CP (cross polarization)/MAS (magic angle spinning) solid state NMR spectra were recorded at room temperature in solid phase and DMSO-d_6 solution. The NMR experiments were performed at room temperature on Bruker Avance DSX-300 spectrometer, operating at 75.47 MHz frequency at ^{13}C, equipped with MAS probehead using 4 mm ZrO_2 rotors. The ^{13}C CP/MAS spectra were recorded with 1 ms contact time, the repetition delay was 10 s and the spectral width was 25 kHz. The ramped amplitude (RAMP) shape pulse was used during the cross-polarisation and TPPM decoupling during the acquisition. The sample was spun at 8.4 kHz, and 3.4 kHz and 4k data points FID's were accumulated. The sample of glycine was used for setting the Hartman–Hahn condition for CP/MAS and as the shift reference ($\delta = 176.04$ ppm C=O).

To generate radicals the samples of CTZ, HCTZ, ATZ, BTZ and FSE in the amount of 50 mg, were exposed to γ-irradiation from a ^{60}Co source to a dose of 150 Gy, at a room temperature. The EPR spectra were recorded at room temperature using a reflection spectrometer operating at 9.4 GHz with 100 kHz field modulation frequency. The magnetic field was controlled with a NMR magnetometer.

3. Calculation details

The quantum chemical calculations were carried out within the GAUSSIAN98^{TM} code [21] running on the CRAY supercomputer in Supercomputer and Network Centre (PCSS) in Poznan. All calculations were performed within the density functional theory (DFT) with three-parameter hybrid functional of Becke B3, in which the local and non-local terms of the correlation functional are provided by the LYP expression [21–24] in the 6–31G* and 6–311+(G2d,p) basis sets. Since the crystallographic structure of HCTZ in solid phase is known [25], the calculations of the chemical shielding were carried out for the crystallographic as well as the optimised geometry. Optimisation of the HCTZ geometry was necessary as the positions of hydrogen atoms calculated in [25] are charged with significant error, e.g. the intermolecular and intramolecular contributions to the second moment of ^{1}H-NMR calculated on the basis of these data were almost twice overestimated [20]. The X-ray geometry of HCTZ was also taken as the initial data for geometry optimisation of the other thiazides. Frequency calculations were performed for all optimised structures to determine stationary points as minima or as saddle points. The NQR quadrupole coupling constants, NMR shielding tensor components and EPR hyperfine coupling constants were calculated at the same level of theory B3LYP. In the case of NMR the gauge-including atomic orbital (GIAO) method [26] was applied. Delocalisation of the electron density was studied on the basis of natural atomic orbitals; (NAO) and natural bond orbitals; (NBO) obtained in the Weinhold–Carpenter procedure [27–30]. The local spin

Table 1
Spectrocopic parameters characterising the electronic structure of thiazides

Compound	R	Biological data SD (mg)[a]	Experimental results[b]				
			^{13}C-NMR		EPR		^{35}Cl-NQR
			$\delta_{C(3)}$ (ppm)	CSA (ppm) ($\eta(-)$)	A_N (mT)	A_H (mT)	e^2Qqh^{-1} (MHz)
HCTZ	–H	25–150	55.7 (54.4[c])	58.88 (0.065)	1.18	2.10	72.958
TCTZ	$-CHCl_2$	5–20	69.3 (70.7[c])	69.3 (0.0)	1.11	3.30	73.800
ATZ	$-CH_2SCH_2CH{=}CH_2$	20–100	63.9 (66.1[c])	63.9 (0.0)	1.13	0.59	72.838
CTZ	–	1000–2000	150.3 (149.7[c])	150.3 (0)	1.18	0.59	74.220
FSE	–	–	–	–	–[b]	–[b]	73.158

[a] Biological activity data from [3,4], complete set of experimental data [14–20].
[b] The lack of stable radical.
[c] Spectra in DMSO-d_6 solution.

values and total charge on a given atom were found using the atoms in molecules theory AIM, proposed by Bader [30], and based on a direct analysis of the scalar $\rho(r)$ and gradient $\nabla\rho(r)$ field of electron density. To calculate the spectroscopic parameters that are not directly determined, e.g. NQR parameters (QCC, η) or NMR parameters (CSA, η), electronegativity, chemical hardness and visualisation of maps (Laplacian of the density).

4. Results and discussion

4.1. Polarisation and delocalisation effects in thiazide molecules

The effect of intramolecular interactions, i.e. electron effects (polarisation and delocalisation), of the substituents on the distribution of electron density in thiazides molecules has been studied by the resonance methods ^{35}Cl-NQR, ^{13}C-NMR, EPR and theoretical DFT methods in [14,15,17], Table 1. The resonance methods ^{35}Cl-NQR [14] and ^{13}C-CP/MAS NMR [15] have been found to be suitable for investigation of the polarisation (inductive) effects and delocalisation (coupling) effects, while EPR can be used to study only polarisation effects in thiazides because the radicals in CTZ and HCTZ are identical [17].

The results obtained from the ^{35}Cl-NQR and ^{13}C-CP/MAS NMR study imply that the delocalisation effects modify the electron density distribution in thiazides much stronger than the polarisation effects. An increase in the quadrupole coupling constant as a result of the delocalisation effects is 3.6%, while as a result of the polarisation effects it does not exceed 1.1%. An increase in the chemical shift as a result of the delocalisation effects is 300% (three times), while as a result of the polarisation effects it is 20%.

The role of the tree types of delocalisation effects taking place in thiazides has been studied in [15]. These three types are:

- π–π coupling between the π-electron systems of the double bond and the benzene ring,
- p–π coupling between the free electron pair of the chloride atom and the π-electron system of the benzene ring,
- tautomeric effects.

The strongest electron delocalisation effects, i.e. the π–π coupling between the π-electron systems of the double bond and benzene ring have been analysed by comparing the spectroscopic parameters for HCTZ and CTZ analogues. Differentiation between the HCTZ and CTZ analogues in the solid state has been made on the basis of the characteristic quadrupole coupling constant (QCC) values on the Cl (6) atom (^{35}Cl-NQR) and chemical shifts at the atoms C(3) and C(6) (^{13}C-NMR). In general, the spectroscopic parameters take much higher values for CTZ than for HCTZ analogues (QCC 2% higher, chemical shifts 3 times higher, CSA 36% higher, the chemical shifts at the carbon atoms C(6) 20% greater).

The p–π coupling between the free electron pair of the chlorine atom and the π-electron system of the benzene ring has been assessed on the basis of ^{35}Cl-NQR spectra and DFT calculation results [14,16]. The mean change in QCC caused by the p–π coupling is 2.83 MHz (NQR). The DFT results proved the co-planarity of the symmetry axes of the p and π orbitals, determining the possibility of this type coupling, and permitted estimation of the asymmetry parameter as 0.09 for all thiazides studied, which is rather high for organic compounds and characteristic of this type of coupling. The sign of the p–π coupling effect was found to depend strongly on the substituent. It is negative for the substituents $-CH_2SCH_2Ph$ or $-CH_2SCH_2CH{=}CH_2$, (when the system is of the A–S–D type, with the electron donor (D) separated from the electron acceptor chlorine atom (A) by the spacer (S) benzothiadiazine ring). It is positive for $-CHCl_2$ when the system is of the A–S–A type of strongly electron acceptor groups (A) separated by the spacer benzothiadiazidine ring.

The assessment of the weakest delocalisation effect, that is the tautomeric effect related to the presence of two forms 2H and 4H of CTZ analogues in the solid phase, was possible on the basis of a correlation of the experimental results with the DFT calculation results. The 4H form with a hydrogen atom at N(4), characterised by higher QCC on the

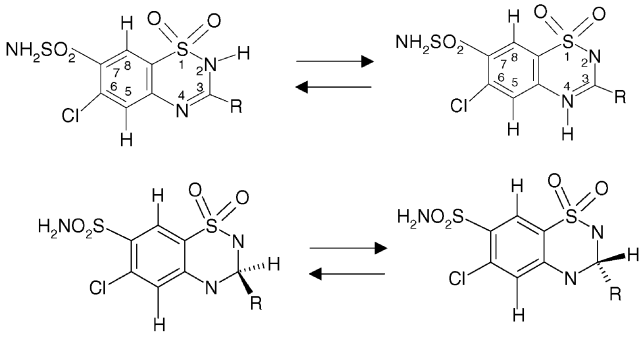

Fig. 2. Possible tautomers of CTZ analogues (2H and 4H) and conformations of HCTZ analogues (*anti* and *syn*).

chlorine atom Cl, ^{35}Cl-NQR, and a smaller chemical shift at C(3) and C(6), ^{13}C-NMR, proved much more stable. As shown in [14,15] differentiation between the two tautomeric forms is possible on the basis of ^{13}C-NMR and ^{35}Cl-NQR in solid phase, Fig. 2. Identification of tautomeric forms on the basis of ^{13}C-NMR is relatively well documented in literature [31–35], whereas there are only a few works on the application of NQR for that purpose [36–38].

In [14,15] a global measure of delocalisation effects was the hydrogen atom deficit index used in mass spectroscopy and found to be correlated with the spectral parameters of ^{35}Cl NQR and ^{13}C-CP/MAS NMR. Taking into regard the effects of delocalisation, the compounds in the ground state were clearly separated into two groups, while in the radical state [14,15] an increase in the delocalisation effects in a system was related to a drastic decrease in the quantum yield of radiation and the absence of radicals in BTZ and FSE.

The results obtained by the resonance methods (^{35}Cl-NQR, ^{13}C-CP/MAS NMR, EPR) have proved that the polarisation effects (in thiazides transferred through bonds) give weaker modification of the electron distribution density and the character of the modification depends on the electron donor or acceptor properties of the substituent. According to decreasing electron donor properties, the substituents in thiazides can be ordered as (resonance methods):

1. At the liquid nitrogen temperature:

$$-CH_2SCH_2Ph > -CH_2SCH_2CH{=}CH_2 > -H > -CHCl_2$$

2. At room temperature:

$$-CH_2SCH_2Ph > -H > -CH_2SCH_2CH{=}CH_2 > -CHCl_2$$

The substituents –CH_2SCH_2Ph and –$CH_2SCH_2CH{=}CH_2$ are electron donors, –$CHCl_2$ is an electron acceptor at 77 K, –CH_2SCH_2Ph is an electron donor, –$CH_2SCH_2CH{=}CH_2$ and –$CHCl_2$ are electron acceptors at room temperature. The fact that –$CH_2SCH_2CH{=}CH_2$ appears as an electron donor at 77 K and an electron acceptor at 293 K, stimulated the need to study the molecular dynamics of thiazides.

The ^{35}Cl-NQR results given in [14] imply that the polarisation effect caused by a substituent is independent of whether it is a fragment of an aromatic or an aliphatic system. The substituents –CH_2SCH_2Ph and –$CH_2SCH_2CH{=}CH_2$ are electron donors and –$CHCl_2$ is an electron acceptor at 77 K irrespective of whether they make a fragment of thiazide or a simple aliphatic system.

Possible correlations were analysed between the spectroscopic parameters characterising the electronic effects in thiazides (QCC) on the chlorine atom Cl(7), from NQR, chemical shift at C(3), from NMR, and the hyperfine coupling constants, determined from the EPR spectra at room temperature. As the paramagnetic component and the quadrupole coupling constant are defined with reference to the same electron density distribution, a linear dependence between these parameters for the compounds whose mean excitation energies are similar, is not surprising. For thiazides the correlation between the chemical shift at C(3) and the QQC on the chlorine atom is very good—the correlation coefficient 0.95, standard deviation 2.9 (Fig. 3), and, as follows from the UV spectra [39,40], the mean excitation energies are close (differences do not exceed 0.5 eV). Hitherto, a linear relationship between the chemical shift and QQC has been observed only for very small molecules of chloromethanes [41].

Because in thiazides the chemical shift at C(3) and isotropic hyperfine structure constants on C(3) as well as N(2) and N(4) are determined by the σ-electron density distribution, then, under a reasonable assumption of close mean excitation energies, a linear relation between these two parameters is expected, Fig. 4a and b. The correlation between the isotropic constants of hyperfine structure on C(3), N(2) and N(4) and the QQC at the chlorine nuclei is very good (correlation coefficient 0.97 or −0.91, standard deviation 0.49 or 0.02), Fig. 5a and b. The different signs at the correlation coefficients follow from the opposite electronic affects on the carbon and nitrogen atoms. Proportional changes in the spectroscopic parameters determined by the magnetic resonance methods (QCC on Cl(7) from NQR, chemical shift at C(3) from NMR and

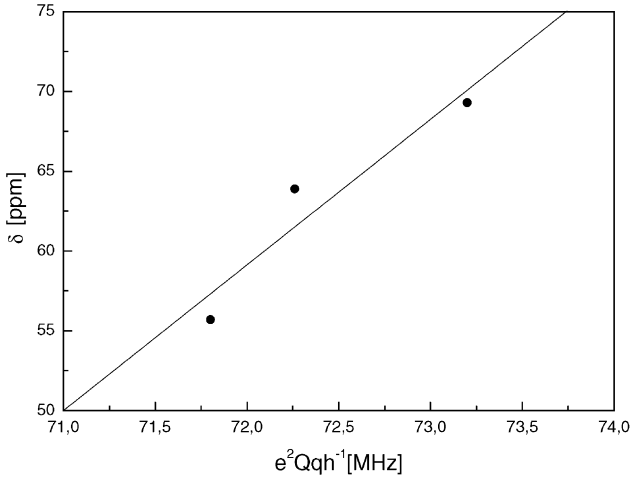

Fig. 3. The correlation between chemical shift on C(3) and quadrupole coupling constant on chlorine atom Cl.

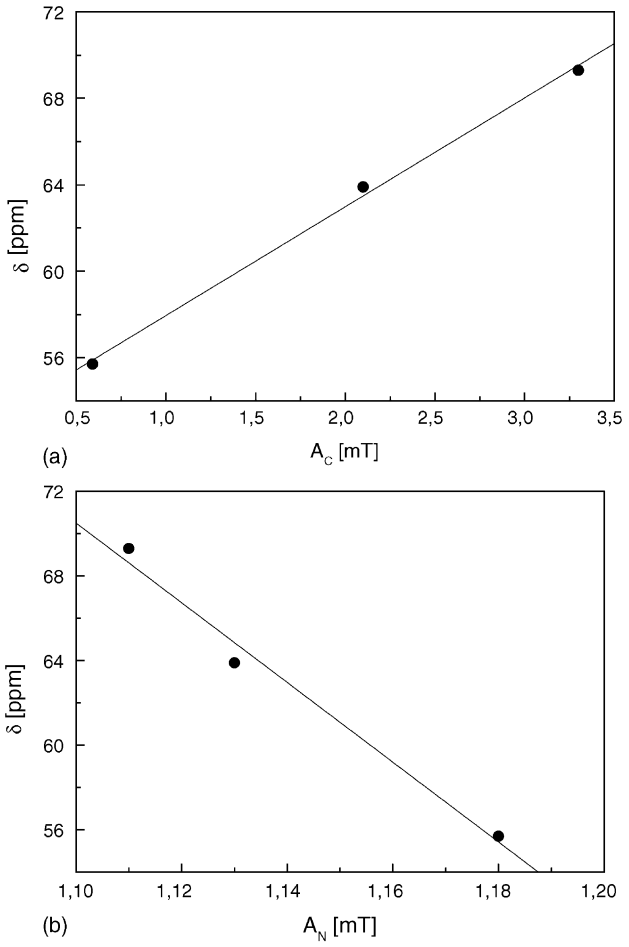

Fig. 4. The correlation between: (a) chemical shift on C(3) and isotropic hyperfine structure constants on C(3); (b) chemical shift on C(3) and isotropic hyperfine structure constants on N(2) and N(4).

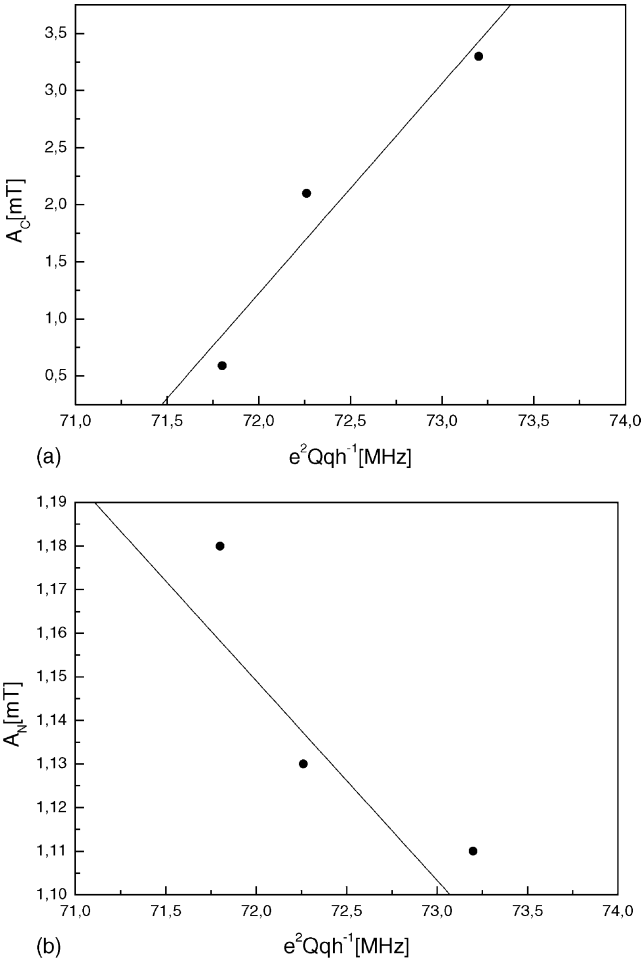

Fig. 5. The correlation between: (a) isotropic hyperfine structure constants on C(3) and quadrupole coupling constant on chlorine atom Cl; (b) isotropic hyperfine structure constants on N(2) and N(4) and quadrupole coupling constant on chlorine atom Cl.

hyperfine coupling constant from EPR) $\delta \sim A_{iso} \sim e^2 Qqh^{-1}$ under the assumed close mean excitation energies of ATZ, TCTZ and HCTZ at room temperature, mean that in these molecules in the ground and radical states the electron density distribution is similar and determined by the polarisation effects. This conclusion is particularly important for interpretation of EPR data and suggests that the delocalisation effects in thiazides are long-range.

The relative changes in the spectroscopic parameters induced by the polarisation effects did not exceed: 1%-NQR (QCC on Cl(7)), 20%-NMR (chemical shift at C(3)) and 6%-EPR (hyperfine coupling constant) at the accuracies of 0.008%-NQR, 0.17%-NMR and 0.85%-EPR, which means that the most sensitive in this type of study is NQR, while the least sensitive is EPR.

4.2. Reproduction of different spectroscopic parameters by DFT

The resonance methods give information on changes in the electron density distribution in thiazide molecules induced by the electronic effects at the sites of particular local probes (chlorine nuclei (^{35}Cl-NQR), carbon nuclei (^{13}C-NMR) and radicals at C(3) (EPR)), however, they are not able to inform about a global distribution of electron density in the molecule. These data are obtained from the calculations of the electronic structure in the ground state for molecular systems and solid state systems within the so-called density functional theory (DFT).

Because of the absence of structural data for thiazides, the geometry of the molecules was optimised by the DFT methods [16]. It required analysis of tautomeric forms (CTZ analogues) or two different conformations corresponding to possible arrangements of the substituents with respect to the plane of the ring (HCTZ analogues).

The energetically preferred were these forms (4H) and conformers (substituent in front of the plane in ATZ, substituent behind the plane in TCTZ) for which the dipolar moment and spatial size were the smallest.

It was shown in [14,16] that the HCTZ structure optimised by DFT was close to the crystallographic structure (correlation coefficient 0.762 and standard deviation 1.3) which is essential from the point of view of the spectroscopic

parameter modelling. The above conclusion is confirmed by a very good correlation between the available experimental IR of thiazides (CTZ and HCTZ) [18] with those calculated by DFT [14]. The short bond N(4)–C(10) 1.367 Å obtained for HCTZ agrees with that determined from X-ray diffraction 1.344 Å. For the other thiazides in the optimised structure this bond is also short (1.374 Å for ATZ, 1.372 Å for TCTZ, 1.391 Å for CTZ and 1.374 Å for BTZ). According to Dupont et al., [25] the presence of a shortened N(4)–C(10) bond in HCTZ can be explained by the resonance structures, however, the occurrence of such structures has not been confirmed by the results of charge distribution. Taking into account the fact that the length of the N(4)–C(10) bond determined from the X-ray study is smaller that that following from the optimisation, and the atom N(4) is endowed with a considerable negative charge, the bond shortening seems to be a result of formation of intermolecular hydrogen bonds involving N(4). This supposition is confirmed by measurements of hydrophobic properties [42,43], indicating facility of hydrogen bond formation by SO_2 and NH, additionally confirmed by the crystallographic data for HCTZ [25] and a successful monitoring of hydrophobic properties of thiazides at the ring position 6, with the chlorine atom. The highest electron density and electrostatic potential occur in the region N(2)–C(3)–N(4), especially on N(4) of the thiazides studied.

As follows from DFT results [16], charge distribution in the thiazide molecules (NPA) and NBO analysis, the polarisation effects caused by a substitution at C(3) bring the greatest changes in the electron density on C(3) directly connected to the substituent and nitrogen atoms N(2) and N(4). The electron and delocalisation effects lead to a decrease in the negative charge at the carbon atom and to an increase in the negative charge at the nitrogen atoms. The substitution leads first of all to an increase in the population of the free electron pair of the nitrogen atom N(4) and a decrease in the population of double bonds in which N(4) is involved, then to an increase in the population of the double bond C(9)=C(10) and a decrease in the population of the double bonds C(5)=C(6) and C(7)=C(8). Changes in the electron density distribution caused by a substitution at C(3) are effectively transferred through the benzothiadizine ring onto the chlorine atom at C(6), and the free electron pair of the nitrogen atom N(4) plays a key role in the process. The sulphonamide group at C(7) of the ring is practically insensitive to electron effects. According to the results of the NBO analysis, the stability of CTZ analogues is mainly due to the interaction of the free electron pair of the nitrogen atom N(4) with the anti-bonding π orbitals of the double bonds ($n_{N4}=\pi^*_{N4–C3}$ and $n_{N4}=\pi^*_{C9–C10}$) [16]. The HCTZ analogues are stabilised by the interactions of the free electron pair of N4 with the anti-bonding π orbital of the double bond ($n_{N4}=\pi^*_{C9–C10}$) and the bonding π orbital with the anti-bonding π* orbital ($\pi_{C7–C8}=\pi^*_{C9–C10}$). With respect to a decreasing energy of stabilisation of the interaction of the free electron pair on N(4) with the anti-bonding π* orbital of the double bond $n_{N4}=\pi^*_{C9–C10}$, the thiazides studied can be ordered as CTZ > BTZ and HCTZ > TCTZ > ATZ. This confirms a great role of the free electron pair on N(4) in thiazides. Changes in the charge on the chlorine atom caused by the substitution are very small and therefore, to reveal the electron density redistribution in the vicinity of chlorine a topological analysis of electron density was applied, using the laplassian [14] being a scalar derivative of the field gradient and hence indicating regions of locally increased or decreased electron density. The changes in the electron density in the vicinity of the chlorine atom in CTZ, caused by the delocalisation effect were found to be restricted to the electron pairs [14], which is confirmed by the p–π coupling. In TCTZ the electron density of the free electron pairs of the chlorine atom at C-6 of the ring is delocalised onto the C–Cl bond and the electron density is redistributed from the C–Cl bond onto the benzothiadizine ring. In ATZ and BTZ the electron density is delocalised from the C–Cl bonds onto the free electron pairs of the chlorine atom at C-6 of the ring and then redistributed from the ring onto the C–Cl bond [14].

Analysis of the structure of the molecular orbitals [16], including the highest occupied and the lowest unoccupied ones (HOMO and LUMO), has proved that in HCTZ analogues the main source of reactive electrons is the nitrogen atom N(4), while in CTZ analogues the nitrogen atoms N(4) and N(2). From among the HCTZ analogues the best electron acceptor is TCTZ. The ordering of thiazides with respect to the HOMO–LUMO energy differences, hardness and electronegativity, according to Parr, is the same as that obtained on the basis of the spectroscopic results. The double degeneration of the HOMO–LUMO orbitals leading to the two reaction pathways (LUMO and LUMO-1) for BTZ has explained why it is impossible to obtain a stable radical in this system.

The spectroscopic parameters including the QCC values, asymmetry parameter chemical shift, chemical shift and the tensor of its anisotropy and the asymmetry parameter of the anisotropy tensor, were calculated on the same level of the theory and with the use of the same functional and basis set for which the geometrical structure of the compounds was optimised.

In [14–16] it was established that the QCC values, chemical shifts and chemical shielding anisotropy CSA were very well reproduced by the same DFT method, i.e. with the same functional B3LYP and basis set 6–311 + G(d,p)). However, because of strong intermolecular interactions, the asymmetry parameters of the CSA tensor for thiazides were not well reproduced. The calculated parameters of QCC asymmetry for thiazides cannot be compared with the experimental data, because the former are close to the values obtained for a compound of a similar structure–chlorobenzene. The quality of reproduction of the QCC anisotropy parameters is affected by the intermolecular interactions (hydrogen bond formation), as shown by the results obtained in [36–38].

The calculated dependence of the resonance frequency as a function of the torsional angle [8,20] confirmed that the –NH_2 group rotation had little influence on the ^{35}Cl-NQR resonance frequency, because the frequency averaged as a result of this rotation was close to that in the absence of rotation, and changes in the resonance frequency due to a possible hindered rotation of substituents distant from the quadrupole nucleus: –$CHCl_2$ or –$CH_2SCH_2CH{=}CH_2$ about the axis of the bond C(3)–C (of the substituent) were negligibly small. Moreover, as follows from the IR spectra analysis, the vibration of the lowest wavenumber activated for ATZ is the vibration of the whole molecule about its axis of inertia. The greatest dynamics showed the substituent –$CH_2SCH_2CH{=}CH_2$, which explains the small slope of the temperature dependence of the NQR resonance frequency for ATZ [20].

4.3. The electronic structure and biological activity of thiazides

Literature gives many contrasting opinions on the electron density distribution and reactive sites in the thiazide molecules. Shinagawa et al., [44] relate the biological activity of thiazides with the π-electron density on the nitrogen atoms N(2), N(4) and the carbon atom C(7), Wohl [45,46] supposes that it is determined by the positive charge on the nitrogen atom N(4). The rotational symmetry of the substituent, according to Cragoe [2], is a consequence of the hydrophobic properties of the substituent, according to Beyer, Baer [47] to chlorouretic properties of thiazides, according to Orita it is related to the formal charge on the atom at C-7 [48] and according to Toplis–to the parameters of octanol–water division [49].

These are mostly theoretical works based on the results of calculations performed by simplified methods (Hückel method [45,46], semi-empirical CNDO/2 [48]), and only a few experimental reports on the physico-chemical properties of thiazides (parameters of octanol–water division [49]). In the recent review papers on thiazides [5–13] this problem is left unsolved and the authors emphasise that the mechanism of the thiazides activity is still unknown. In the papers published till 1997, the diuretic activity of thiazides was related to inhibition of carbonic anhydrase (CA) [44]. As follows from recent studies, the diuretic properties of thiazides are determined by the reflux absorption of sodium and chlorine based on the inhibition of the protein 115 kDa (built of 1021 amino acids) of the Na^+–Cl^- co-transporter sensitive to thiazides (TSC, NCC or SLC12A3) [6–8].

In 1999, Chang [50] proposed a model explaining the biological activity of thiazides in which he assumed that thiazides compete with chlorine in the process of bond formation with the co-transporter Na^+–Cl^-.

Also it was experimentally proved [6] that the Na^+–Cl^- co-transporter with a thiazide instead of chlorine cannot permeate through cell membranes. The model was developed by Lloyd who used the CellML [51]. However, the Chang model did not explain the way of the bonding of a thiazide with the Na^+–Cl^- co-transporter. The problem is important as, according to the recent results, thiazides are successful in therapy of osteoporosis and the interest in them is growing [9,10,12,13].

The correlated spectroscopic and quantum-mechanical research, reported in this work, has permitted a comprehensive analysis of the electron density distribution and identification of the reactive sites in thiazide molecules. Finally it has been established that the biological activity of thiazides is determined by the electron density distribution in the region N(2)–C(3)–N(4), which has been concluded from the results of the experimental study on the chlorine atom at the position C(7) [1] and on all carbon atoms [2] in the ground state, and in direct neighbourhood of the radical (i.e. on the chlorine atoms N(2) and N(4), the chlorine atom from –$CHCl_2$ and C(3) from the radical [14,19]) and the DFT calculations [16]. The delocalisation effects decrease biological activity of thiazides, whereas the inductive effects modify it (decrease or increase depending on the substituent being an electron donor or acceptor), and the density changes of the free electron pair at N(4) play the most important role N(4) [14–16]. As reported in [14,15,19] with increasing single dose of administered drug, (corresponding to its decreasing biological activity) the spectroscopic parameter describing the polarisation effect also decreases. The same conclusions have been drawn irrespectively of the spectroscopic parameter analysed: quadrupole coupling constant, chemical shift, hyperfine coupling constants. Although the number of points presented in Fig. 6 illustrating the character of the dependence of spectroscopic parameters on the single dose is small (the small number of compounds studied) the tendency is clear. The changes in QCC constant detected by NQR at the chlorine atom Cl(7) localised far from the substitution site, show the same tendency as the changes in the chemical shifts at C(6) and

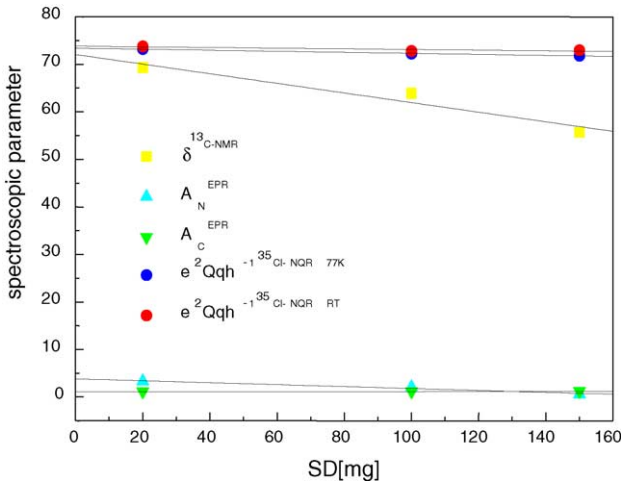

Fig. 6. Spectroscopic parameters vs. biological activity of the thiazides studied expressed by the single dose (SD).

C(3)detected by ^{13}CP/MAS NMR and the changes in the isotropic hyperfine coupling constants at C(3) detected by EPR. However, the most sensitive to the polarization (inductive) electronic effects is the ^{35}Cl-NQR spectroscopy.

The appearance of the delocalisation effect on coming from HCTZ to CTZ is accompanied by a significant increase in the spectroscopic parameter, which induces a significant increase in the single dose and thus a drastic decrease in the biological activity, Table 1.

A sufficient support of the conclusions comes from the fact that analogous relations are obtained when analysing spectroscopic parameters obtained from three different methods (NMR, EPR, NQR) with biological activity. The intercorrelation of the spectroscopic parameters for thiazides has been analysed in detail on the basis of the spectroscopic theories and confirmed by DFT calculations [52].

As indicated by the results of a biological study, the weakest from among the thiazides studied is CTZ, while the strongest diuretic is TCTZ with the electron acceptor group $-CHCl_2$ at C(3), whose replacement by hydrogen (HCTZ) or $-CH_2SCH_2CH{=}CH_2$ (ATZ) significantly reduces its biological activity. The Parr reactivities determined for thiazides by the DFT method in [3] suggest the highest chemical reactivity of TCTZ and the lowest of CTZ, while the electronegativity calculated for TCTZ is close to that of a chlorine atom, which in the light of the Chang model, explains the highest biological activity of TCTZ. The results given in [14–17,19] suggest that the source of the reactive electrons is the nitrogen atom N(4), and because of the type of radical, formed upon γ-irradiation, the most active site in the molecule is the carbon atom C(3) and these atoms play the main role in the process of the thiazide bonding with the Na^+–Cl co-transporter.

5. Conclusions

A comparative analysis of the experimental results obtained by the resonance methods (NQR, NMR, EPR) and the results of DFT calculations performed for the thiazides studied leads to the following conclusions.

Analysis of the influence of the electronic effects (polarisation and delocalisation) on the electron density distribution in the thiazide molecules has shown the following.

1. The spectroscopic parameters: quadrupole coupling constants QCC on the Cl(6) atom (^{35}Cl-NQR) and chemical shifts at the carbon atoms C(3) and C(6) (^{13}C-NMR) permit a differentiation of the CTZ and HCTZ analogues in solid phase. The results of DFT calculations supplementing the experimental results additionally permit identification of 2H and 4H forms of CTZ analogues and conformation of HCTZ analogues.
2. The resonance methods ^{35}Cl-NQR and ^{13}C-CP/MAS NMR provide information on the polarisation (inductive) effects and delocalisation (coupling) effects, while the EPR method, because of the type of radical, provides information on the polarisation effects in thiazides only.
3. On the basis of the results obtained by the methods NQR, NMR, EPR and DFT, according to the decreasing electron donor properties the substituents in the thiazides studied can be ordered as:

a. At the liquid nitrogen temperature:

$$-CH_2SCH_2Ph > -CH_2SCH_2CH{=}CH_2 > -H > -CHCl_2$$

b. At room temperature:

$$-CH_2SCH_2Ph > -H > -CH_2SCH_2CH{=}CH_2 > -CHCl_2$$

4. At 77 K the substituents $-CH_2SCH_2Ph$ and $-CH_2SCH_2CH{=}CH_2$ are electron donors, $-CHCl_2$ is electron acceptor, while at room temperature $-CH_2SCH_2Ph$ is an electron donor, $-CH_2SCH_2CH{=}CH_2$ and $-CHCl_2$ are electron acceptors.
5. The polarisation effect generated by a substituent is independent of whether it is a part of an aromatic or aliphatic system.
6. The electron density distribution in the thiazides studied is stronger modified by the delocalisation than by the polarisation effect.
7. The spectroscopic parameters (QCC on the chlorine atom Cl(7), NQR, chemical shift at C(3), NMR and the hyperfine coupling constant, EPR) determined at room temperature are very well correlated, which means that they describe the same electronic effects and that the effects in the ground and radical state are similar.
8. The NQR is the most sensitive from among the resonance methods applied.

The conclusions regarding the application of the DFT methods for reproduction of different spectroscopic parameters and modelling of the intra- and intermolecular interactions are given below.

1. Changes in the electron density distribution caused by a substitution at C(3) are well transferred through the benzothiadizine ring onto the chlorine atom at C(6), and the free electron pair of the nitrogen atom N(4) plays a key role in the process.
2. The DFT results have shown that the greatest electron density and the highest electrostatic potential occur in the region of the bonds N–C–N, in particular at N(4).
3. The CTZ analogues are stabilised by the interactions of the free electron pair of N(4) with the anti-bonding π-orbitals of the double bonds ($n_{N4}{=}\pi^*_{N4–C3}$ and $n_{N4}{=}\pi^*_{C9–C10}$). The HCTZ analogues are stabilised by the interactions of the free electron pair of N(4) with the anti-bonding π-orbital of the double bond ($n_{N4}{=}\pi^*_{C9–C10}$) and the bonding π-orbital with the anti-bonding π* orbital ($\pi_{C7–C8}{=}\pi^*_{C9–C10}$). According to decreasing energy of stabilisation of the free electron pair of N(4) interaction with the anti-bonding π*-orbital of the double

bond $n_{N4}=\pi^*_{C9-C10}$ the thiazides can be ordered as CTZ > BTZ and HCTZ > TCTZ > ATZ.

4. The DFT methods very well reproduce the quadrupole coupling constants (irrespective of the type of isotope), chemical shifts and their anisotropy (CSA), but, because of strong intermolecular interactions, they poorly reproduce the asymmetry parameters CSA for thiazides.
5. The calculations of the quadrupole coupling constants should be performed taking into account not only all electrons but also the effect of electrons correlation.
6. The pseudopotential method (ECP) calculations lead to the quadrupole coupling constants much lower than their experimental values, the difference is the greater the heavier the atom. The differences come from the different sign contributions to the potential, and thus to the electric field gradient components used in determination of QCC.
7. The accuracy of QCC values determination from the density matrix (Townes-Dailey model) is 5%.

The conclusions concerning the correlations of the electronic effects with the biological activity of thiazides are as follows.

1. The spectroscopic parameters (NMR, EPR, NQR) characterising the electronic effects are very well correlated with biological activity of the thiazides studied.
2. The biological activity of thiazides is determined by the electron density distribution in the region N(2)–C(3)–N(4), decreased by the delocalisation effects and modified (increased or decreased) by the polarisation effects depending on the substituent.
3. In the aspect of increasing the biological activity, the electron acceptor substituents are favoured, which is consistent with the Chang model, according to which thiazides bind with the co-transporter Na^+–Cl^- instead of Cl.
4. The results of the study suggest that the source of reactive electrons is the nitrogen atom N(4) and it plays the main role in the process of thiazide bonding with the co-transporter Na^+–Cl.

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