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Chemical modification of human albumin at cys_{34} by ethacrynic acid: structural characterisation and binding properties[1]

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Abstract

Derivatization of the free cys_{34} in human albumin, which is reported to occur under physiological conditions, has been performed in vitro by reaction of the protein with ethacrynic acid. This modification has been investigated by mass spectrometry and circular dichroism. Ethacrynic acid has been proven to bind human albumin either covalently and non-covalently. This post-translational modification does not determine significant changes in the secondary structure of the protein, as shown by the comparable circular dichroism spectra of the native and the modified proteins. Furthermore, the binding properties of the human albumin samples have been investigated by circular dichroism and equilibrium dialysis. The affinity to the higher affinity binding sites does not change either for drugs binding to site I, like phenylbutazone, or to site II, like diazepam, while a small but significant increase has been observed for bilirubin, known to bind to site III. Nevertheless significant decreases of the affinity at the lower affinity binding sites of the modified protein were observed for both drugs binding to site I or to site II.

Keywords: Cys_{34} modified HAS; Ethacrynic acid; Protein binding; Mass spectrometry; Circular dichroism

1. Introduction

The consensus is that, for most drugs, the portion in plasma is the most biologically active. Thus plasma protein binding influences both pharmacokinetics and pharmacodinamics, the human albumin (HSA) being the most abundant carrier in serum. This protein presents several binding sites for the reversible binding of endogenous and exogenous molecules [1–6]. In some cases the ligands bind covalently to HSA and these non-enzymatic post-translational modification reactions have been demonstrated to occur in physiological conditions [7,8]. Glycosylation by glucose [9–15], acylation by aspirin [16–20] and oxidation of cys_{34} [2,7,21–24] surely represent the

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more widely investigated modifications of HSA. Among these, the binding of endogenous or exogenous compounds to the cys_{34}, the sole free cysteine residue on HSA, are much less well characterised, despite intense interest over many years. HSA is indeed heterogeneous including molecules containing a free SH group in cys_{34} and components where this SH group is oxidised or masked by cysteine or glutathione. The amount of free cys_{34} components is variable with age and in various disease states such as chronic renal failure, and liver cirrhosis [25–27]. Recently, the covalent binding of the anti-rheumatic drug bucillamine to HSA has been investigated with the aim of studying the mechanism of reaction of drugs with the thiol moiety [28]. Further the binding of ethacrynic acid (ETA), a drug used as a diuretic and as a treatment for glaucoma, has been used to selectively modify HSA when it is anchored to a silica matrix [29]. ETA also binds HSA non-covalently and at least two binding sites are involved [30–32]. Both covalent [2,23,29] and non-covalent [30–32] binding of ETA determine significant changes of the binding properties of the protein. However no structural evidence was obtained for a modification at cys_{34}. The interest in the non-enzymatic post-translational modification of HSA resides also in the immunological consequences of the covalent bindings [33,34]. Indeed the hapten hypothesis of drug hypersensitivity has been proposed also for thiol-containing drugs [35].

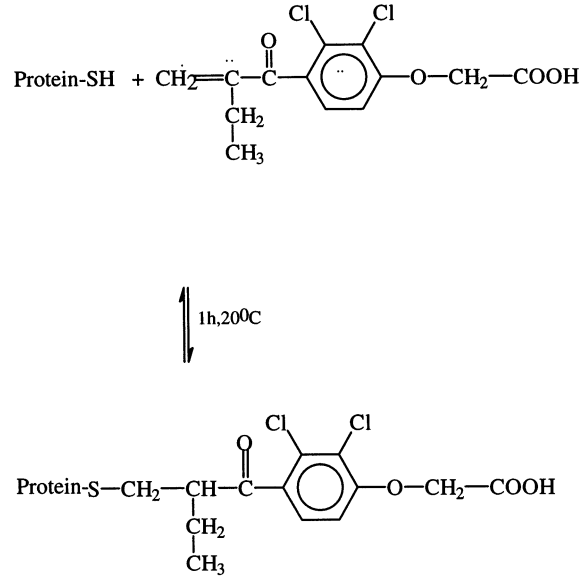
Here we report the structural characterisation of the cys_{34} modified HSA obtained by reaction in vitro of the protein with ETA (Scheme 1).

Recombinant human albumin (rHA) was used for this study to ensure the homogeneity of the samples that are essential for a reliable mass spectrometry investigation. This methodology was employed to determine the number of ETA residues bound to HSA, either covalently or non-covalently. Further the binding properties of the cys_{34} modified albumin, as compared to those of the native one, were investigated by circular dichroism (CD) and equilibrium dialysis. Drugs were chosen that selectively bind to specific binding areas, i.e. site I (warfarin-azapropazone binding site), site II (benzodiazepines binding site) and site III (bilirubin binding site) [4]. Finally the influence of the chemical modification on the secondary structure of the protein was investigated by CD at high energy, where the electronic transitions of the peptide bonds occur.

2. Materials and methods

2.1. Materials

HSA (fraction V, essentially fatty acid free), was supplied by Sigma (St. Louis, MO) and used without further purification. rHA, obtained by expression of HSA gene in the yeast *Saccharomyces cerevisiae*, was supplied by Delta Biotechnology (Castle Court, Nottingham, UK) as solution 25% (w/v) in 145 mmol l^{-1} sodium chloride, 0.16 mmol sodium octanoate g^{-1} albumin and 15 mg l^{-1} Tween 80. rHA was defatted before use by a slightly modified version of the Chen procedure [36]. Aliquots of 2 ml rHA were dialysed against a H_2O/1-propanol (80/20) mixture, with 1 g charcoal l^{-1}, for 1 h. The pH of the suspension was adjusted at pH 3 with HCl 0.1 N. Subsequent washings were performed changing the 1-propanol concentration from 20 to 1%. The last three washings were performed with phos-



Scheme 1.

phate buffer 10 mM at pH 4.6, 7.4, and 7.4, respectively. Spectroscopic analysis (absorption and CD) does not reveal significant differences between serum and recombinant albumins.

HSA concentration was determined by measuring the absorbance at 279 nm ($\varepsilon^{279} = 32180$, according to Elwell's method) [37].

ETA {[2,3-dichloro-4-(2-methilene-1-oxobutyl) phenoxy]acetic acid} and phenylbutazone (4-butyl-1,2 - diphenyl - 3,5 - pyrazolidinedione) were supplied by Sigma. Bilirubin was purchased from Fluka Chemika (Buchs, Switzerland). Diazepam (7-chloro-Nl-methyl-1,3-dihydro-5-phenyl-2H-1,4 benzodiazepin-2-one) was kindly provided by Professor A. Lucacchini, Instituto Policattedra di Discipline Biologiche, Facoltà di Farmacia, Università di Pisa, Italy. All reagents were analytical grade, and they were used without further purification.

2.2. Modification of HSA by ETA

The modification of HSA was carried out following, with minor changes, a literature procedure reported for the modification of bovine serum albumin [38]. In practice HSA (or rHA) was dissolved in potassium phosphate buffer 10 mM pH 7.4 and fivefold excess molar of ETA was added. The solution was stirred at room temperature for 1 h, then dialysed against potassium phosphate buffer 10 mM pH 7.4 for 20 h.

2.3. Instruments

Mass spectrometry measurements were carried out on a Perkin-Elmer SCIEX API III triple quadrupole mass spectrometer (Sciex, Thomhill, Canada) equipped with an articulated ionspray interface. The operative parameters were set as follows: ionspray voltage, 5.5 kV; orifice voltage, 90 V; scan range, 1400–2200 u; scan speed, 8.42 s; resolution > 1 u. The spectra were acquired in multichannel acquisition (MCA) mode summing 20 scans. Solutions at molar ratios rHA/ETA 1/1, 1/2 and 1/3 (previously prepared and dialysed) were diluted in $H_2O/CH_3CN/HCOOH$ 50/50/0.2 in order to obtain a concentration of 40–50 μM. The resulting solution was analysed by continuous infusion into the source by a Harvard model 22 syringe pump (Harvard Apparatus, South Natick, MA) at 5 μl min^{-1} flow rate.

Electrophoretic experiments were performed with isoelectrofocusing (IEF) method. The running gel was prepared as follows: 3.1 ml distilled water, 0.65 ml bis-acrylamide 30%, 1 ml glycerol 25%, 0.2 ml ampholine with pH gradient 3–10; the solution was degassed for 15 min, then 70 μl ammonium persolfate and 5 μl tetramethylenediamine were added. Runs were performed with a Biorad Miniprotean II (Bio-Rad Laboratories, Hercules, CA) at the following voltages: 100 V for 15 min, 200 V for 15 min and 450 V for 1 h.

CD and absorption spectra were recorded using a Jasco J-600 spectropolarimeter (Jasco, Tokyo, Japan) and a Varian Cary 4E spectrophotometer (Varian, Sidney, Australia); both instruments were interfaced to personal computers to acquire and elaborate data. All measurements were carried out at room temperature in quartz cells and the spectra were recorded with the same instrumental parameters (sensitivity, scan rate, time constant) to reduce errors. For experiments at high energy (180–250 nm) rHA, HSA and HAS–ETA solutions were 15 μM in phosphate buffer, the cell path length was 102 μm.

The affinity constants were determined by CD spectroscopy, measuring the induced CD spectra for [marker]/[HSA] 1/1 complexes at different concentrations. HSA concentration ranged from $10^{-3}/10^{-4}$ to $10^{-6}/10^{-7}$ M and cell path length correspondingly varied from 0.01 to 10 cm to maintain an optical density between 0.2 and 0.4. All the samples were prepared from the same stock solution in phosphate buffer pH 7.4 to minimise errors. The concentration of each stock solution was checked by UV absorption. Thus, in practice, the value of the affinity constant, together with that of the $\Delta\varepsilon$ for the protein/drug complexes, can be obtained by simply measuring the value of the induced CD signal upon the total concentration of the protein [39]. The equilibrium constant of the complex,

$$K = [\text{HSA} - \text{drug}]/[\text{HSA}][\text{drug}] = [\text{LP}]/[\text{P}][\text{L}] \quad (1)$$

assumed with a 1:1 stoichiometry and the Lambert and Beer law, applied to the circular dichroism,

$$CD = A_L - A_R = [LP]\, l\, \Delta\varepsilon \quad (2)$$

can indeed be combined, according to the Benesi–Hildebrand treatment [40], to give the following linear equation:

$$\frac{p}{\sqrt{\frac{CD}{l}}} = \frac{1}{\Delta\varepsilon}\sqrt{\frac{CD}{l} + \frac{1}{\sqrt{K\Delta\varepsilon}}} \quad (3)$$

where $p = [P] + [LP] = [L] + [LP]$; CD, measured circular dichroism; l, cell pathlength; $\Delta\varepsilon = \varepsilon_L - \varepsilon_R$, where ε_L and ε_R are the molar absorption coefficients for left and right circular polarised light, respectively, at the absorption wavelength.

For equilibrium dialysis experiments, [marker]/[HSA] complexes at different ratios (0.25/1, 0.50/1, 0.75/1, 1/1, 1.25/1, 1.50/1, 1.75/1, 2/1, 2.50/1, 3/1 and 4/1) were prepared in phosphate buffer, pH 7.4. HSA concentration was constant (90 μM), while the marker concentration varied according to the required ratio. Aliquots of complexes (l.2 ml) were dialysed for 72 h at 4°C against 0.2 ml phosphate buffer inserted in a cellophane dialysis tubing Spectrapore (Spectrum, Medical Industries, Houston, TE). The free marker concentration was determined by HPLC, using a standard curve for each marker. HPLC runs were performed on a Jasco 880-PU chromatograph (Jasco, Tokyo) equipped with a Jasco 875-UV spectrophotometer (Jasco, Tokyo, Japan) and interfaced with a personal computer. Chromatographic conditions were:

- phenylbutazone: the column was a C8 Aquapore RP-300 (7 μm, 100 × 4.6 mm) (Brownlee, Applied Biosystems, Foster City, CA), mobile phase CH_3OH/ CH_3COOH/ heptanesulfonic acid 1 mg ml^{-1} 60/40/0.1, flow 1 ml min^{-1}, detection 240 nm, 100 μl loop injection.
- diazepam: the column was a Ultropac Lichrosorb RP-18 (5 μm, 250 × 4 mm) (E. Merck, Darmstadt, Germany), mobile phase CH_3OH/ H_2O 70/30, flow 0.5 ml min^{-1}, detection 254 nm, 100 μl loop injection.

Binding constants were calculated by using the Scatchard's equation:

$$r = \sum_{i=1}^{j} \frac{n_i \cdot K_i \cdot [L]}{1 + K_i \cdot [L]} \quad (4)$$

where r is the average number of moles of ligand bound per mole of HSA, n_i and k_i represent the number of binding sites and the corresponding association constant for the i-th binding class, and [L] is the concentration of free ligand.

3. Results and discussion

rHA was used for this study because the heterogeneity of commercially available HSA makes difficult its structural characterisation. In particular little differences in the molecular weight arising from the ETA binding could not be reliably detected by mass spectrometry if the protein sample is not homogeneous.

3.1. Characterisation of the modified protein

Ionspray mass spectrometry results efficiently give evidence of the binding of ETA to the protein, to determine the nature of this binding as well as the stoichiometry of the resulted complexes. The results obtained with rHA are shown in Fig. 1(a) and they are compared with those related to the modified samples of the protein obtained by incubation of rHA in phosphate buffer solution at 37°C for 1 h with an equimolar amount of ETA (Fig. 1b) or with a threefold molar excess of the derivatizing agent (Fig. 1c). The modified sample obtained using a 1/1 [ETA]/[rHA] molar ratio in the derivatization reaction shows a prevalent peak at 66734 Da, which has to be assigned to the modified protein sample with a single residue of ETA bound to rHA (Fig. 1b). The sample obtained using a 3/1 [ETA]/[rHA] molar ratio in the incubation experiment, shows two molecular peaks, one corresponding to the sample with an ethacrynic residue bound to rHA (66734 Da) and the other corresponding to two residues bound to the protein (67034 Da, Fig. 1c). Defatted samples of the protein were used for these experiments. The derivatizing procedure, i.e. incubation of rHA with an equimolar amount or with an excess of ETA, was carried out also using a sample of the non-defatted protein. This protein was used because fatty acids compete with the noncovalent binding sites of the ETA. Thus the

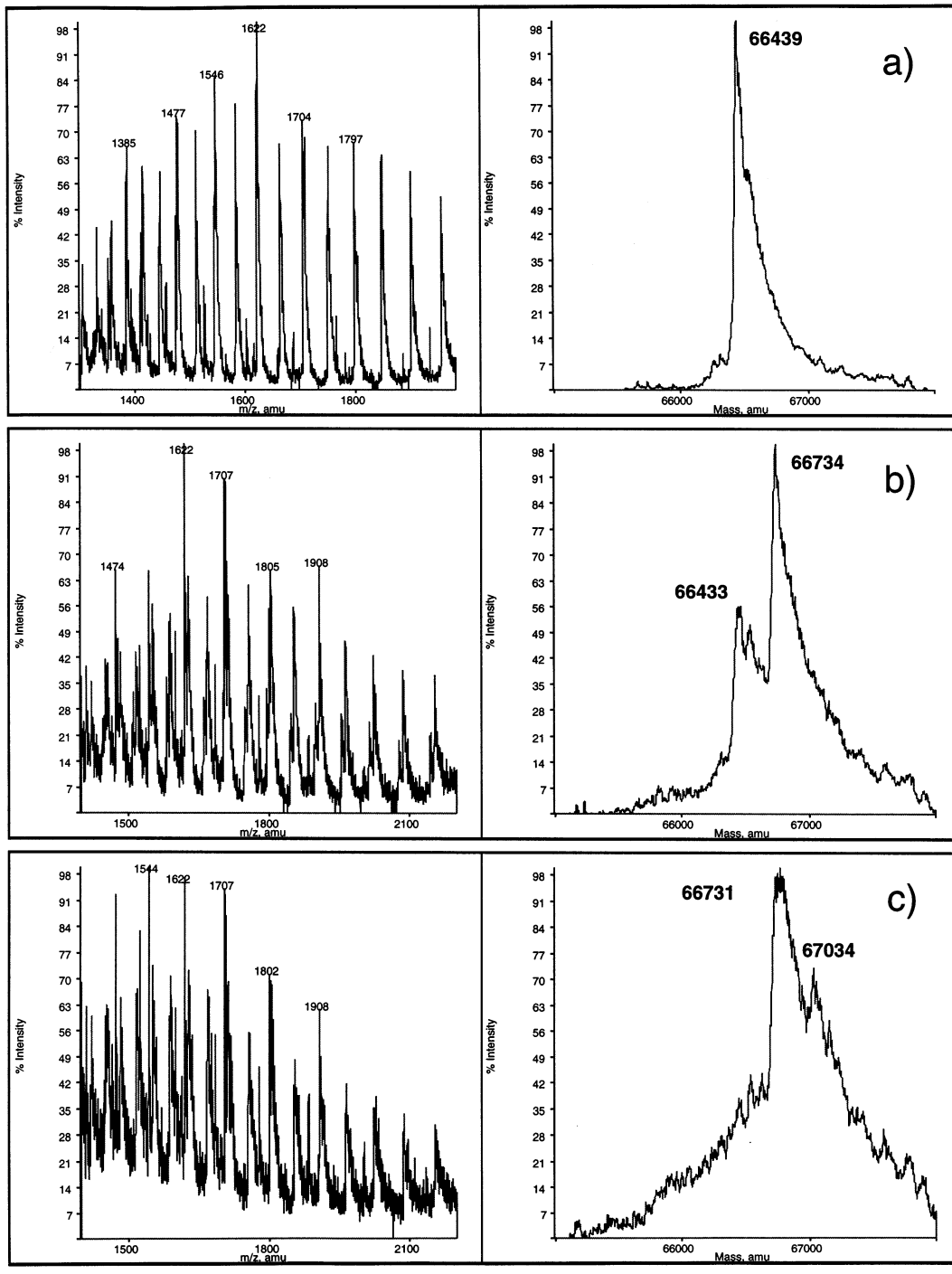


Fig. 1. Mass spectra of defatted samples of rHA in $H_2O/CH_3CN/HCOOH$ 50/50/0.2. (a) Native protein (40 μm); (b) after incubation with an equimolar amount of ethacrynic acid (50 μm); (c) after incubation with a threefold molar excess of ethacrynic acid (50 μm).

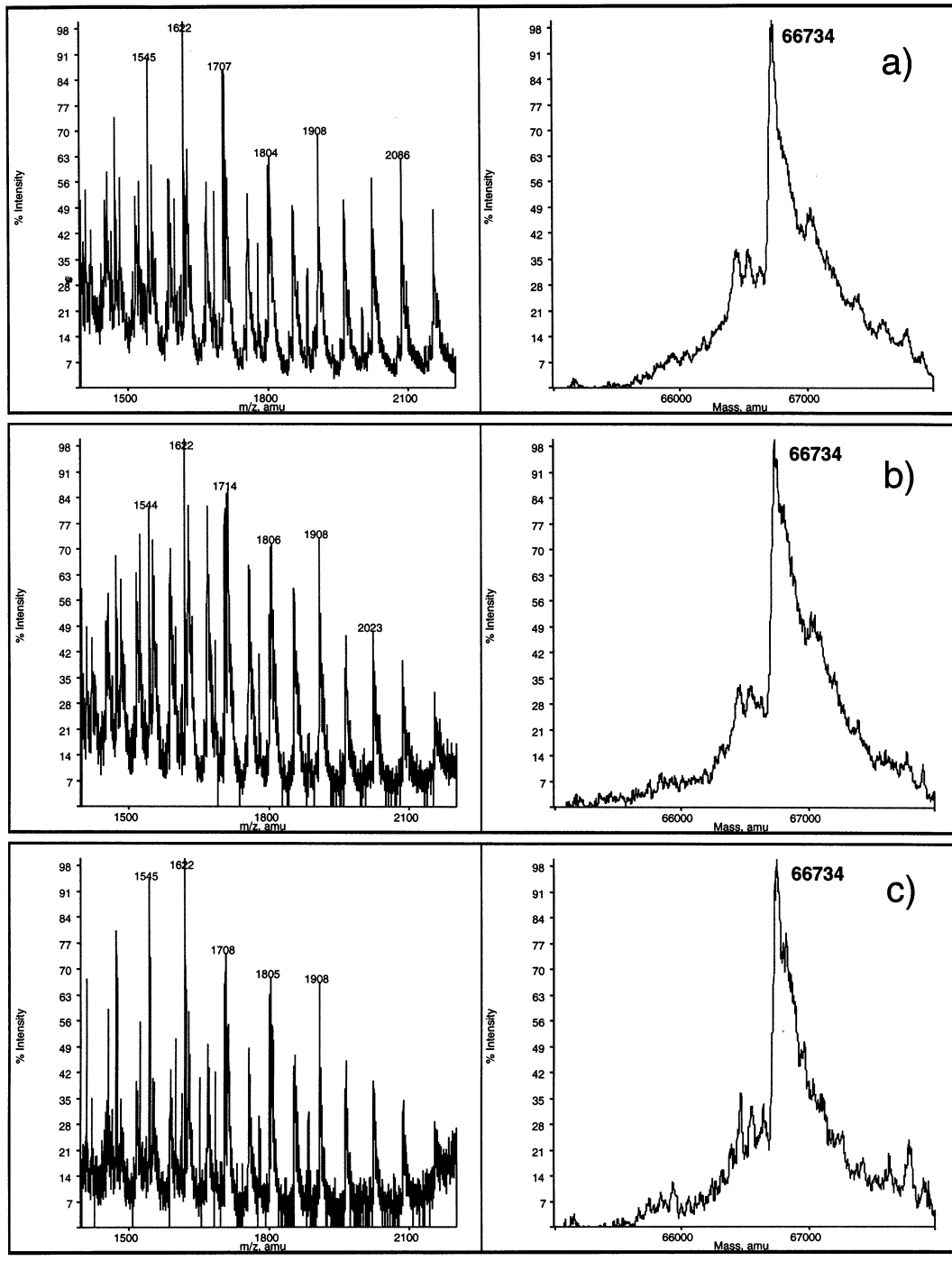


Fig. 2. Mass spectra of modified rHA samples in $H_2O/CH_3CN/HCOOH$ 50/50/0.2. (a) non-defatted rHA after incubation with an equimolar amount of ethacrynic acid (45 μm); (b) non-defatted rHA after incubation with a threefold molar excess of ethacrynic acid (50 μm); (c) defatted rHA after incubation with a threefold molar excess of ethacrynic acid and extensive dialysis (50 μm).

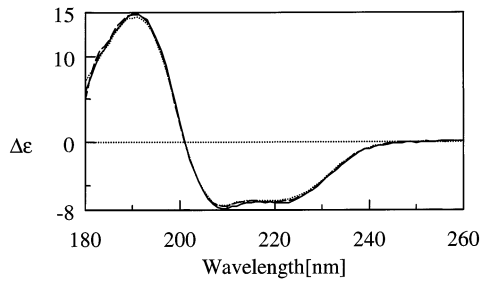


Fig. 3. CD spectra of rHA (solid line), HSA (dotted line) and cys_{34} modified albumin (dashed line). [rHA], 15 μM in phosphate buffer 20 mM, pH 7.4, cell 102 μm.

modification could be more selective for the cys_{34} residue under these experimental conditions. Both samples of modified protein obtained by incubation with equimolar amounts or threefold excess of ETA, show a molecular peak corresponding to the binding of one residue of ETA to rHA (Fig. 2a, b). The same result was obtained when the modified protein with two residues of ETA, previously discussed (Fig. 1c), was extensively dialysed against phosphate buffer (Fig. 2c). These results strongly support the hypothesis that one residue of ETA binds covalently to the protein as suggested by the stability of the 1/1 adduct after extensive dialysis, while the removal of the second residue suggests that it binds non-covalently to rHA.

The cys_{34} derivatized protein sample was also characterised by the IEF technique. The modified protein has a lower isolectric point (4.6 ± 0.1) with respect to that of the starting protein (5.1 ± 0.1). This result is in agreement with the nature of the derivatizing agent which adds the contribution of the free carboxylic group as well as of two electronegative chlorine atoms at the phenyl moiety (Scheme 1).

Furthermore the influence of the derivatization on the secondary structure of the protein has been investigated by CD. The analysis has been carried out in the region where the electronic transitions of the peptide bond occur (180–240 nm) [41]. The CD spectra of rHA is typical for a protein with relatively high α-helical content with a positive maximum at about 190 nm ($\Delta\varepsilon_{max}$, 13.7) and two negative bands centred at about 208 ($\Delta\varepsilon_{max}$, -7.4) and 222 nm ($\Delta\varepsilon_{max}$, -6.8) (Fig. 3). The CD spectrum of the cys_{34} derivatized sample of rHA is, in practice, superimposable to that of the native sample (Fig. 3), this suggesting that no significant change in the secondary structure of the protein occurred after chemical derivatization of the protein. This result is not surprising taking into account that cys_{34} is located at the amino-end portion of the protein.

3.1.1. Protein binding properties

The modified sample of rHA has been characterised for its binding properties as compared to those of the native one. The interest arises from the fact that serum protein binding influences both pharmacokinetics and pharmacodynamics. Thus it is essential to determine if and how the chemical modification affects the affinity of endogenous or exogenous compounds which bind to specific binding areas.

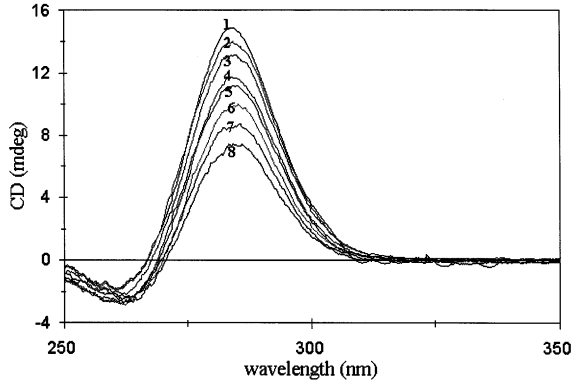
3.1.2. CD difference spectra

The measure of the CD difference spectra, which are due to the induced CD signal of the ligand when it binds to the protein, gives direct information on the ligand/protein complex in terms of stoichiometry and stereoselectivity of binding. The observed CD signal is indeed proportional to the amount of bound ligand, and this property has been used to determine drug–protein binding parameters ([42–44]). Here the method is applied to determine the binding properties of the modified protein, in terms of affinity constant, with respect to the native one.

The quantitative analysis of the CD data is relatively easy when the ligand is achiral and a single site interaction on the protein is responsible for the induced CD. The CD signal will then arise from the complexed ligand and the result obtained will reflect selectively the binding site producing the induced CD signal [45]. Three markers were used: phenylbutazone, diazepam and bilirubin, which are known to bind at site I, site II and site III, respectively. These ligands show induced CD signals at wavelengths where the contribution of the protein is negligible or absent. The change of the lowest energy CD induced band of the complexed drug at different dilution of the [HSA]/[drug] adduct in a 1/1 molar ratio, allows the dissociation constant, related to the stereoselective binding site,

to be determined. The intensity of the induced CD spectra indeed decreases by decreasing the concentration of the 1/1 [drug]/[protein] complex, depending on its dissociation constant. The data obtained in the complexation of phenylbutazone and diazepam are reported in the Figs. 4 and 5, respectively.

The values of $\Delta\varepsilon_{max}$ of the complex and of its dissociation constant can be obtained by applying Eq. (3) (see experimental section) and plotting $[HSA]/(CD/l)^{1/2}$ against $(CD/l)^{1/2}$ (Fig. 4b, Fig. 5b). The analysis of the CD data has been performed looking at the value of the CD band at



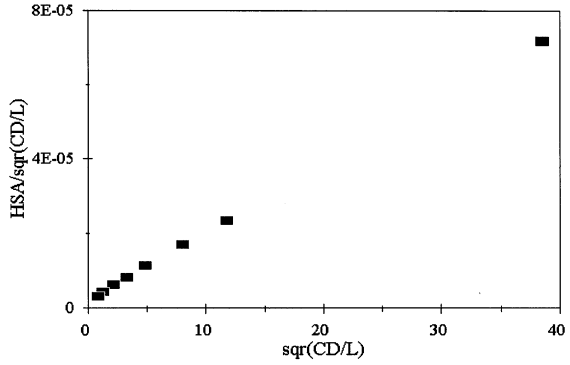
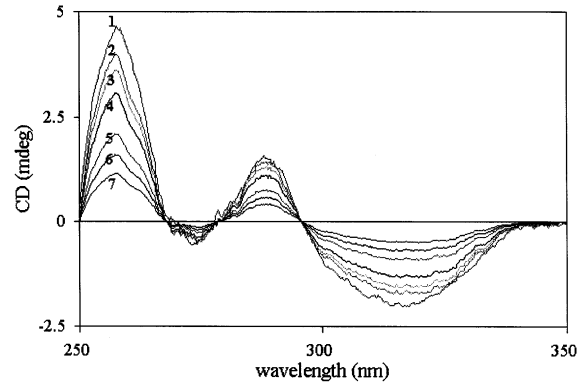


Fig. 4. (a) CD difference spectra of [phenylbutazone]/[HSA] 1/1 complexes in phosphate buffer 20 mM, pH 7.4. (1) $[HSA] = 8 \times 10^{-4}$ M, 0.01 cm cell; (2) $[HSA] = 1.6 \times 10^{-4}$ M, 0.05 cm cell; (3) $[HSA] = 8 \times 10^{-5}$ M, 0.1 cm cell; (4) $[HSA] = 4 \times 10^{-5}$ M, 0.2 cm cell; (5) $[HSA] = 1.6 \times 10^{-5}$ M, 0.5 cm cell; (6) $[HSA] = 8 \times 10^{-6}$ M, 1 cm cell; (7) $[HSA] = 1.6 \times 10^{-6}$ M, 5 cm cell; (8) $[HSA] = 8 \times 10^{-7}$ M, 10 cm cell. (b) Linear fitting for 1/1 [phenylbutazone]/[HSA] complexes, 285 nm.



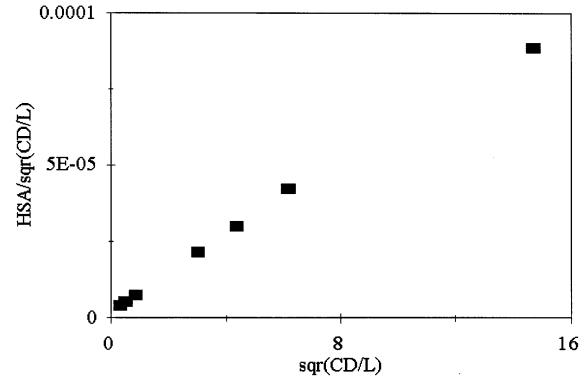


Fig. 5. (a) CD difference spectra of [diazepam]/[HSA] 1/1 complexes in phosphate buffer 20 mM, pH 7.4. (1) $[HSA] = 1 \times 10^{-3}$ M, 0.01 cm cell; (2) $[HSA] = 2 \times 10^{-4}$ M, 0.05 cm cell; (3) $[HSA] = 1 \times 10^{-4}$ M, 0.1 cm cell; (4) $[HSA] = 5 \times 10^{-5}$ M, 0.2 cm cell; (5) $[HSA] = 1 \times 10^{-5}$ M, 1 cm cell; (6) $[HSA] = 2 \times 10^{-6}$ M, 5 cm cell; (7) $[HSA] = 1 \times 10^{-6}$ M, 10 cm cell. (b) Linear fitting for 1/1 [diazepam]/[HSA] complexes, 315 nm.

lower energy in order to have minimum or no interference by the contribution to the CD arising from the protein. Thus the band at 285 nm has been selected in the case of phenylbutazone, the band at 315 nm in the case of diazepam and the band at 420 nm in the case of bilirubin. The obtained values of the affinity constants are reported in the Table 1 and compared with those of the native protein. The results suggest that the chemical modification does not significantly affect the affinity to the high affinity binding sites for phenylbutazone and diazepam while a moderate but significant increase has been observed in the

Table 1
Affinity constants determined by circular dichroism and equilibrium dialysis

	Native rHA		Modified rHA	
	CD	Dialysis	CD	Dialysis
DZP	$K_1 = 6.3\ (\pm 0.2) \times 10^5$	$K_1 = 6\ (\pm 0.3) \times 10^5$	$K_1 = 6.2\ (\pm 0.4) \times 10^5$	$K_1 = 6\ (\pm 0.2) \times 10^5$
	----------	$K_2 = 5.3\ (\pm 0.2) \times 10^4$	----------	$K_2 = 2\ (\pm 0.4) \times 10^4$
PBU	$K_1 = 4.6\ (\pm 0.2) \times 10^5$	$K_1 = 4.4\ (\pm 0.3) \times 10^5$	$K_1 = 4.8\ (\pm 0.3) \times 10^5$	$K_1 = 4.5\ (\pm 0.2) \times 10^5$
	----------	$K_2 = 8\ (\pm 0.3) \times 10^4$	----------	$K_2 = 4.5\ (\pm 0.4) \times 10^4$
BIL	$K_1 = 1.8\ (\pm 0.3) \times 10^6$	----------	$K_1 = 2.8\ (\pm 0.1) \times 10^6$	----------

DZP, diazepam; PBU, phenylbutazone; BIL, bilirubin.

case of the high affinity binding site of bilirubin (Table 1).

The applied difference CD method allows a definite characterisation of the stereospecific binding that induces a preferential enantiomeric conformation in the achiral ligand. Compared to other widely used methods, it allows the study of a stereospecific binding site, disregarding secondary or non-stereospecific binding on HSA. These results, however, do not exclude changes in the binding properties to low affinity binding sites of the modified protein. Indeed lower affinity has been proved for drugs binding to site I or to site II by affinity chromatography. Significant decrease of the retention time, which reflects the affinity binding, was observed in the analysis of drugs on a human albumin based HPLC column before and after the chemical derivatization of cys_{34} by in situ reaction with ETA [29]. Thus equilibrium dialysis experiments were carried out in order to confirm these data.

3.1.3. Equilibrium dialysis

The equilibrium dialysis experiments allowed the determination of the binding properties of phenylbutazone and diazepam to the native and to the cys_{34}-modified samples of human albumin. The Scatchard equation for two sites was applied and the affinity constant values for the two drugs were determined for both the high and low affinity binding sites. Bilirubin was not used for this investigation because the instability of this compound in solution does not allow reliable dialysis experiments to be carried out. Almost the same values of the affinity constants, for the native and the derivatized protein samples, were obtained in the case of the high affinity binding sites of phenylbutazone and diazepam. These values were in agreement with those determined by the difference CD method (Table 1). On the contrary, significant differences were obtained for affinity constant values at the lower affinity binding sites of the two protein samples. A decrease of about 60 and 40% was observed, after derivatization, for the affinity binding constant of phenylbutazone and diazepam to the low affinity binding site (Table 1).

4. Conclusions

Samples of cys_{34} modified human albumin have been obtained by reaction of the protein with ETA. The derivatized protein samples were characterised for their structure by mass spectrometry and CD. These methodologies appear well suited to the determination of structural changes in HSA, as the derivatization of the cys_{34} residue. In particular an ETA residue covalently bound to the human albumin was observed, while one or two additional molecules of the drug were non covalently bound. The cys_{34} derivatized protein maintains the secondary structure of the native albumin as suggested by the superimposition of their CD spectra.

Further the affinity constants of phenylbutazone, diazepam and bilirubin, at least for the high affinity binding sites, remained unchanged for the modified protein with respect to the native one. On the contrary, a significant reduction in the

affinity constants of phenylbutazone and diazepam for the low affinity binding sites in the case of the derivatized albumin was observed.

Thus significant changes in the binding properties of HSA can occur when the protein is highly derivatized at cys_{34}. The possibility of having a greater insight into the characterisation of drugs or metabolites when they are covalently bound to HSA is of general interest, taking into account that the ligand–protein conjugated could be often the cause of adverse hypersensitivity reactions observed for many drugs.

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References

[1] W.E. Muller, U. Wollert, Pharmacology 19 (1979) 59–67.
[2] T.Jr. Peters, Serum albumin, Adv. Protein Chem. 37 (1985) 161–245.
[3] U. Kragh-Hansen, Pharmacol. Rev. 33 (1981) 17–53.
[4] K.J. Fehske, W.E. Mueller, U. Wollert, Biochem. Pharmacol. 30 (1981) 687–692.
[5] X.M. He, D.C. Carter, Nature 358 (1992) 209–215.
[6] D.C. Carter, J.X. Ho, Adv. Protein Chem. 45 (1994) 153–203.
[7] J.J. Harding, Adv. Protein Chem. 37 (1985) 247–334.
[8] U. Kragh-Hansen, Dan. Med. Bull. 37 (1990) 54–87.
[9] J.F. Day, S.R. Thorpe, J.W. Baynes, J. Biol. Chem. 254 (1979) 595–597.
[10] R. Dolhofer, O.H. Wieland, FEBS Lett. 103 (1979) 282–286.
[11] C.E. Gunthrow, M.A. Morris, J.F. Day, S.R. Thorpe, J.W. Baynes, Proc. Natl. Acad. Sci. 76 (1979) 4258–4261.
[12] R.L. Garlick, J.S. Mater, J. Biol. Chem. 258 (1983) 6142–6146.
[13] J.W. Baynes, S.R. Thorpe, M.H. Murtiashaw, Methods Enzymol. 106 (1984) 88–98.
[14] N. Shaklai, R.L. Garlick, H.F. Bunn, J. Biol. Chem. 256 (1984) 3812–3817.
[15] N. Okabe, N. Hashizume, Biol. Pharm. Bull. 17 (1994) 16–21.
[16] R.S. Farr, G.P. Rodnan, E.C. Lasser, J. Clin. Invest. 40 (1961) 1037–1039.
[17] R.S. Farr, R.T. Reid, P. Minden, J. Clin. Invest. 45 (1966) 1006–1012.
[18] R.S. Farr, J. Allergy 45 (1970) 321–331.
[19] J.E. Walker, FEBS Lett. 66 (1976) 173–175.
[20] C. Bertucci, A. Viegi, G. Ascoli, P. Salvadori, Chirality 7 (1995) 57–61.
[21] J.S. Stamler, O. Jaraki, J. Osborne, D.I. Simon, J. Keaney, J. Vita, D. Singel, R. Valeri, J. Loscalzo, Proc. Natl. Acad. Sci. USA 89 (16) (1992) 7674–7677.
[22] C.F. Shaw, A.A. Isab, J.D. Hoeschele, M. Starich, J. Locke, P. Schulteis, J. Xiao, J. Am. Chem. Soc. 116 (1994) 2254–2260.
[23] H. Vorum, K. Fisker, M. Otagiri, A.O. Pedersen, U. Kragh-Hansen, Clin. Chem. 41 (11) (1995) 1654–1661.
[24] J. Christodoulou, P.J. Sadler, FEBS Lett. 376 (1995) 1–5.
[25] S. Era, Acta Sch. Med. Univ. Gifu. 39 (1991) 6–24.
[26] S. Era, K. Kuwata, H. Imai, K. Nakamura, T. Hayashi, M. Sogami, Biochim. Biophys. Acta 1247 (1995) 12–16.
[27] A. Van Der Vliet, C.E. Cross, B. Halliwell, C.A. O'Neill, Methods Enzymol. (Biothiols Part A) 251 (1995) 448–455.
[28] R. Narazaki, M. Hamada, K. Harada, M. Otagiri, Pharm. Res. 13 (1996) 1317–1321.
[29] C. Bertucci, I.W. Wainer, Chirality 9 (1997) 335–340.
[30] K.J. Fehske, W.E. Müller, Pharmacology 32 (1986) 208–213.
[31] N. Takamura, A. Haruta, H. Kodama, M. Tsuruoka, K. Yamasaki, A. Suenaga, M. Otagiri, Pharm. Res. 13 (7) (1996) 1015–1019.
[32] E.M. Sellers, J. Koch-Weser, Clin. Pharmacol. Ther. 11 (1970) 524–529.
[33] M. Yvon, P. Anglade, J.M. Wal, Fed. Eur. Biochem. Soc. 263 (2) (1990) 237–240.
[34] B. Magi, B. Marzocchi, L. Bini, C. Cellesi, A. Rossolini, V. Pallini, Electrophoresis 16 (1995) 1190–1192.
[35] B. Park, N. Kitteringham, Drug Metab. Rev. 22 (1990) 87–144.
[36] R.F. Chen, J. Biol. Chem. 242 (1967) 173–181.
[37] M.L. Elwell, J.A. Shellman, Biochim. Biophys. Acta 494 (1977) 367–383.
[38] D.A. Koechel, S.A. Smith, E.J. Cafruny, J. Pharm. Exp. Ther. 203 (2) (1977) 272–282.
[39] G. Ascoli, C. Bertucci, P. Salvadori, J. Pharm. Sci. 84 (1995) 137–141.
[40] H.A. Benesi, J.H. Hildebrand, J. Am. Chem. Soc. 71 (1949) 2703–2707.
[41] N. Greenfield, J.D. Fasman, Biochem. 8 (1969) 4108–4116.
[42] A. Rosen, Biochem. Pharmacol. 19 (1970) 2075–2081.
[43] J.H. Perrin, D.A. Nelson, J. Pharm. Pharmacol. 25 (1973) 125–130.
[44] N. Roodsorp, I. Sjokolm, Biochem. Pharmacol. 25 (1976) 2141–2145.
[45] C. Bertucci, P. Salvadori, E. Domenici, in: Hassan, Y. Aboul-Enein, I.W. Wainer (Eds.), The Impact of Stereochemistry on Drug Development and Use, Wiley, New York, 1997, pp. 521–543.