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Short communication

Influence of octanoic acid on the reversible protein binding of ketorolac enantiomers to human serum albumin (HSA): comparative liquid chromatographic studies using a HSA chiral stationary phase

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Abstract

The retention of ketorolac enantiomers on a human serum albumin (HSA)-based HPLC chiral stationary phase (CSP) was investigated to assess the utility of immobilized protein for probing the binding of (*R*)- and (*S*)-ketorolac to native HSA. Results from the chromatographic study were compared with enantiomorph binding data obtained from HSA ultrafiltration experiments conducted both in the presence and absence of the medium chain-length fatty acid octanoic acid. Without octanoic acid in the mobile phase containing 10% propan-2-ol in 20 m*M* phosphate buffer at pH 6.5, racemic ketorolac was stereochemically resolved with the HSA-CSP with large enantiomeric capacity factors [106.2 and 28.7 for (*R*)- and (*S*)-ketorolac, respectively]. The inclusion of octanoic acid in the column eluent reduced the capacity factors of both isomers consistent with displacement of drug from HSA binding sites. A reduction in the capacity factor ratio [(*R*):(*S*)] was observed as the octanoate concentration increased from 0.5 to 4.0 m*M*. The percentage unbound of (*R*)- and (*S*)-ketorolac present separately (2.0 μg/ml) in 40.0 mg/ml HSA solution (22°C and pH 7.4) was 0.245% and 0.643%, respectively, and both values increased as a function of increasing octanoate concentration in the HSA solution. A biphasic effect of octanoate on the percentage unbound ratio of (*S*):(*R*) was observed. In light of these findings, it would appear that silica-immobilized HSA is capable of qualitatively probing the enantioselective binding of ketorolac to HSA and moreover, more than one specific ketorolac binding site may exist on the HSA molecule.

1. Introduction

Ketorolac is a nonsteroidal anti-inflammatory drug (NSAID) which is used clinically as a racemate for the short-term management of pain [1]. The non-narcotic analgesic effects of ketorolac are elicited by enantioselective inhibition of prostaglandin biosynthesis; the (*S*)-enantiomer of ketorolac, in common with other chiral NSAIDs, is the pharmacologically active isomer [2]. Recently, we reported that the disposition of ketorolac in man is subject to marked

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enantioselectivity, with approximately a two-fold higher plasma clearance observed for (*S*)-ketorolac compared to its optical antipode following intramuscular administration of the racemate [3]. Elucidation of the in vitro plasma protein binding of ketorolac isomers indicated that these dispositional differences, recorded in terms of total (protein bound plus unbound) drug, are largely accounted for by enantioselectivity of the unbound fractions [4]; ketorolac is classified as a restrictively cleared drug and hence the total clearance of its enantiomers will be dependent on their unbound fractions [5].

Clearly, knowledge of the protein binding of isomers of chiral drugs is important when assessing enantioselective pharmacokinetics. However, with compounds such as NSAIDs which are typically highly bound to human serum albumin (HSA), measurement of unbound drug enantiomers is technically difficult by conventional means [6]. The development and application of a high-performance liquid chromatographic chiral stationary phase (HPLC-CSP) based on immobilized HSA (HSA-CSP) provides an alternative tool for examination of enantioselective aspects of ligand binding to albumin [7–9]. HSA-CSP columns have been reported to broadly retain the binding characteristics of the native protein and upon the addition of specific modifiers to the mobile phase, have provided a means of probing the source of enantioselectivity in the binding of chiral ligands to HSA as well as factors which may alter reversible binding of drugs [10].

In conjunction with a previously validated ultrafiltration method for the elucidation of ketorolac enantiomer binding to HSA [4], we have assessed the suitability of a commercially available HSA-CSP for probing the enantioselective binding of this drug. Moreover, since fatty acids have been shown to modify drug binding to albumin ([11] and references therein), the influence of the medium chain-length fatty acid octanoic acid, on ligand binding both to native and HPLC-CSP immobilized HSA was investigated.

2. Experimental

Racemic ketorolac (unlabelled) was a gift from Syntex Research (Palo Alto, CA, USA). Tritium labelled enantiomers of ketorolac {(*R*)- and (*S*)-5-[3′,4′,5′-$^{3}H_{3}$]-benzoyl-[7-^{3}H]-1,2-dihydro-3*H*-pyrrolo[1,2*a*]pyrrole-1-carboxylic acid}, each of specific activity 3.0 Ci/mmol, were furnished by regiospecific palladium-catalysed exchange of racemic drug with tritiated water followed by HPLC purification and direct resolution [4]. Sodium octanoate and fatty acid-free HSA (Cat. No. A1887) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from BDH (Poole, UK). Chiral HPLC was performed using a Chiral-HSA 100 × 4 mm I.D. column obtained from Chromtech AB (Norsborg, Sweden) and separation of free from protein bound (*R*)- and (*S*)-ketorolac was conducted using a Centrifree ultrafiltration device (Amicon Division, Beverly, MA, USA).

Chromatography was carried out isocratically and at ambient temperature (22°C), using a Waters (Milford, MA, USA) HPLC system incorporating a Model 510 pump, 712 Wisp autoinjector, Model 490 UV-absorbance detector and Millennium version 2.0 Chromatography Manager. The mobile phase was 10% propan-2-ol in 20 m*M* potassium phosphate buffer adjusted to a final pH of 6.5 with sodium hydroxide, in some cases modified with a varying concentration of octanoic acid (0.5–4.0 m*M*). All mobile phases were filtered (0.22 μm) and degassed immediately prior to use and pumped at a flow-rate of 0.8 ml/min. The column eluent was monitored for UV-absorbance at 310 nm following injection of 5.0-μg amounts of (*RS*)-ketorolac. The chromatographic parameters measured were capacity factor (k') defined as $(t - t_0)/t_0$ where t is the retention time of the enantiomer of interest and t_0 is the column void time (determined by injection of water) and α, the resolution factor, calculated as the quotient $k'(R)/k'(S)$ (the capacity factors of (*R*)- and (*S*)-ketorolac, respectively). Parameters were determined in duplicate for each concentration of octanoic acid after equilibration of the col-

umn. With the exception of fatty acid concentration, all conditions were identical such that changes in k' could be solely attributed to the presence of octanoic acid in the mobile phase.

The protein binding of (*R*)- and (*S*)-ketorolac (2.0 μg/ml of each isomer) in HSA (40 mg/ml, 600 μM) in 0.067 *M* phosphate buffer (22°C, pH 7.4) was performed both alone and in the presence (0.5–16.0 m*M*) of octanoic acid. Following the addition of freshly purified and resolved [^{3}H]-labelled enantiomers to HSA solutions, the unbound species were radiometrically detected in ultrafiltrate as previously described [4]. Fractions unbound of each isomer were determined in duplicate at each concentration of octanoic acid after a 30-min pre-equilibration of the HSA solution with the fatty acid.

3. Results and discussion

Racemic ketorolac was stereochemically resolved on the HSA-CSP as depicted in Fig. 1. Diaz-Perez et al. [12] have similarly reported a separation of ketorolac enantiomers using a HSA-CSP. The stereoconfiguration of the first and second eluting enantiomers on the HSA-CSP was confirmed by separate injection of (*R*)- and (*S*)-ketorolac obtained by fractional recrystallization of the diastereomeric salts of cinchonine and cinchonidine, respectively [2]. Consistent with the protein binding data obtained with the ultrafiltration studies, the more retained enantiomer on the HSA-CSP column was indeed the enantiomer with the smaller unbound fraction, i.e. (*R*)-ketorolac. High capacity factors were obtained for both isomers (Fig. 2), consistent with the extensive reversible binding of ketorolac to HSA noted in this study (Fig. 3) and to plasma protein in a previous examination [4]. Increasing concentrations of octanoic acid in the mobile phase dramatically reduced $k'(S)$ and $k'(R)$ (Fig. 2). Parallel studies examining the binding of (*R*)- and (*S*)-ketorolac to native HSA demonstrated reductions in the protein binding of each isomer as a function of increasing octanoic acid concentration (Fig. 3). A biphasic effect of octanoate on the percentage unbound ratio of the enantiomorphs in HSA solution was also observed (Fig. 3).

Preliminary studies confirmed the absence of any isotope effect in the binding of the stereoisomers to the HSA-CSP and subsequently, as distinct from the ultrafiltration binding stüdies, non-radiolabelled drug was used to assess enantiomer binding to the chiral column. We also verified that octanoic acid added to the mobile phase did not permanently affect the immobilized protein by rechromatographing ketorolac after completion of the studies when the effects of octanoic acid were measured. Previously, we have reported [4] precision estimates of $<2\%$ for determining the percentage unbound of each stereoisomer of ketorolac by the method used in this study.

Octanoic acid appeared to displace both ketorolac enantiomers from albumin binding loci as indicated by the HSA-CSP and ultrafiltration studies (Figs. 2 and 3). Accordingly, since medium chain-length fatty acids (including octanoate) together with 2-arylpropionate NSAIDs are thought to bind to site II of the hypothesised specific ligand binding sites of albumin [13–15], it would appear that (*R*)- and (*S*)-ketorolac share this binding site. However, it has been suggested that classical site II occupiers, the 1,4-benzodiazepines, share multiple albumin binding loci and moreover, allosterically interact with the site I binding domain [16,17]. The highly stereoselective binding of ketorolac to HSA observed in this study would appear to involve participation of more than one binding site based on the biphasic effect of increasing octanoate concentration on the percentage unbound enantiomeric ratio. HSA-CSP binding studies also showed a change in the stereoselectivity factor (α) supporting the likelihood of multiple binding sites for ketorolac isomers. In a recent study, octanoic acid was shown to reduce the HSA-CSP retention of a series of NSAIDs with results suggesting that this interaction takes place at more than one binding site [18]. Interestingly, we previously observed a putative allosteric interaction between the long-chain fatty acid, oleic acid, and ketorolac en-

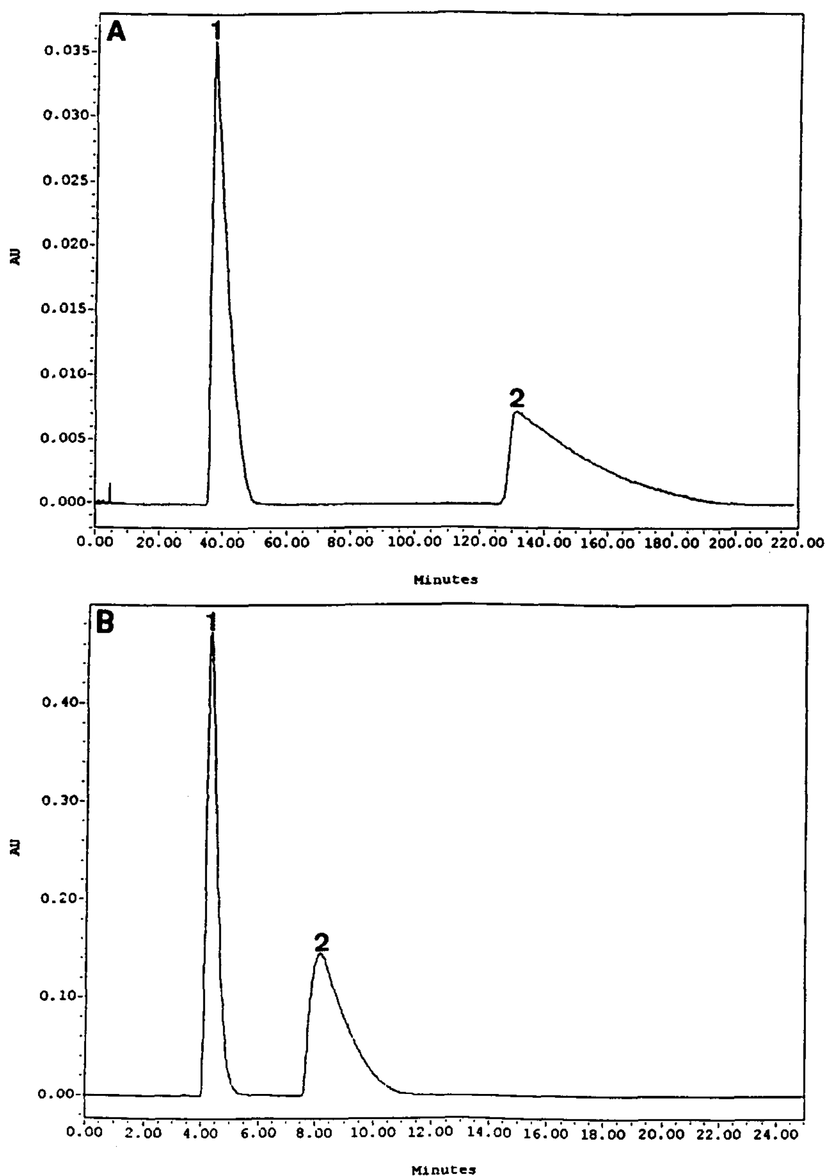

Fig. 1. HSA-CSP chromatograms following injection of 5.0 μg of racemic ketorolac: (A) in the absence of octanoic acid in the mobile phase, and (B) with 4.0 m*M* octanoic acid included in the mobile phase. HPLC conditions are described in Experimental. Peaks 1 and 2 are (*S*)- and (*R*)-ketorolac, respectively.

antiomer binding to HSA [4]. In that study the fraction unbound of (*R*)- and (*S*)-ketorolac was reduced in the presence of oleic acid. This supports some involvement of HSA site I in ketorolac enantiomer binding based on the ability of oleic acid to enhance ligand binding at site I [11].

It would have been desirable to have examined the modification of HSA-CSP binding of ketorolac enantiomers by oleic acid however,

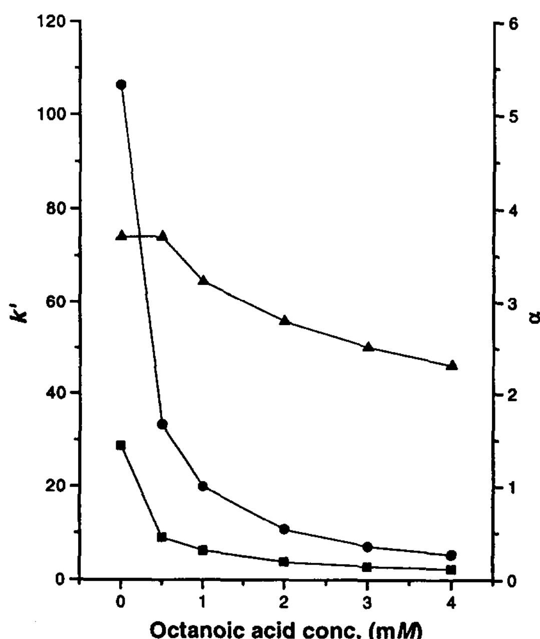

Fig. 2. The capacity factors (left axis) of (R)-ketorolac (●) and (S)-ketorolac (■) and the resolution factor α [$= k'(R)/k'(S)$, right axis] (▲) as a function of octanoic acid concentration in the mobile phase.

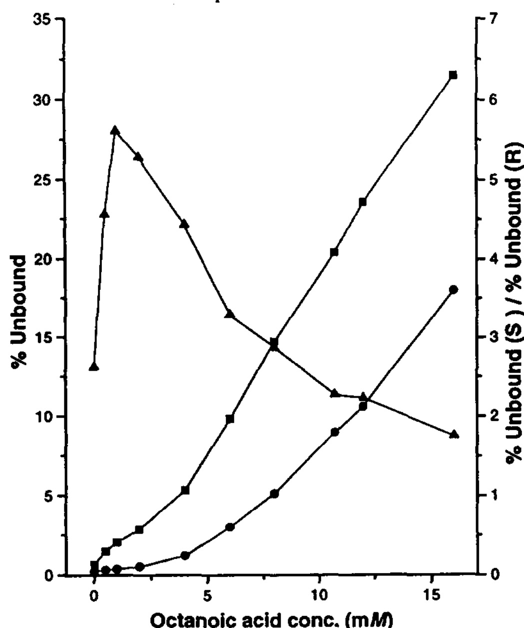

Fig. 3. The percentage unbound of (R)-ketorolac (●) and (S)-ketorolac (■) and the percentage unbound ratio of (S):(R) (▲) following the addition of 2.0 μg/ml of individual enantiomers to 40 mg/ml (600 μM) HSA solution at 22°C and pH 7.4 containing various concentrations of octanoic acid.

such long-chain fatty acids (including their salts) are insoluble in mobile phases compatible with this column chemistry. The possibility of competition between ketorolac enantiomers for HSA binding sites was not investigated here based on a lack of interaction observed previously between the antipodes with in vitro ultrafiltration binding studies [4].

In conclusion, this study confirms that silica-immobilised HSA is capable of probing qualitatively the enantioselective protein binding of ketorolac. Based on our observations of the modification of isomer binding by octanoic acid, the HSA-CSP is a useful indicator of the situation occurring with native protein. Furthermore, these data suggest that more than one specific binding site on albumin may be involved with the reversible binding of ketorolac enantiomers.

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