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Note

Relative capacity factors of some keto bile acids on a reversed-phase high-performance liquid chromatography system

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Although there are a number of reports on the separation and quantitation of the common bile acids by high-performance liquid chromatography (HPLC)[1–3], little information is available on the application of this technique to the separation of keto bile acids. In man these metabolites are produced by oxidation of one or more hydroxyl groups by various intestinal bacteria and are found in relatively high amounts in faecal samples.[4]

We have therefore determined the relative capacity factors of several hydroxyl- and keto-substituted conjugated and unconjugated 5β-cholan-24-oic acids using a reversed-phase HPLC system primarily developed for the separation of the common human bile acids.[3]

EXPERIMENTAL

Materials

All the hydroxyl and keto bile acids studied were 5β-cholan-24-oic acids and were obtained from Calbiochem (Bishops Stortford, U.K.), Steraloids (Croydon, U.K.) and Sigma (Poole, U.K.), or were prepared by enzymic oxidation of these bile acid standards (Tables I–III). The latter was achieved by incubation of bile acid standard solutions with 3α- or 7α-hydroxysteroid dehydrogenase (chromatographically purified grades from Sigma) and β-nicotinamide–adenine dinucleotide (oxidised, grade II, Boehringer, Lewes, U.K.) for 1 h at 37°C in 0.1 *M* pyrophosphate buffer (pH 9.5). Following incubation the reaction mixtures were passed through a Sep-Pak C_{18} cartridge (Waters Assoc., Northwich, U.K.) primed with water. The cartridge was washed with 10 ml of water, the keto bile salts were eluted in 5 ml of methanol and the extracts taken to dryness under nitrogen. The dried extracts were reconstituted in mobile phase and injected directly into the HPLC system. HPLC-grade methanol was from Rathburn Chemicals (Walkerburn, U.K.) and all other solvents and reagents were AnalaR grade from BDH (Poole, U.K.).

HPLC

The HPLC system was composed of modular units supplied by Waters Assoc. and comprised a Model 6000A solvent delivery system, a Model U6K universal injector, a Model R401 differential refractometer and a RCM100 radial compression

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TABLE I

RELATIVE CAPACITY FACTORS (*rk'*; RELATIVE TO CHOLIC ACID) OF HYDROXYL- AND KETO-SUBSTITUTED UNCONJUGATED 5β-CHOLAN-24-OIC BILE ACIDS

Substituents	*rk'*	*Substituents*	*rk'*
3,7,12-Triketo*	0.13	3,6-Diketo**	0.67
3α-Hydroxy-7,12-diketo**	0.16	3-Keto-6β-hydroxy	0.70
3α,12α-Dihydroxy-7-keto**	0.29	3α-Hydroxy-6-keto***	0.73
3α,7α-Dihydroxy-12-keto*	0.32	3-Keto-6α-hydroxy	0.77
3α,6β,7β-Trihydroxy (β-muricholic)**	0.46	3α-Hydroxy-7-keto**	0.85
3-Keto-6β,7β-dihydroxy	0.46	3α,6α-Dihydroxy (hyodeoxycholic)**	0.90
3-Keto-7α,12α-dihydroxy*	0.51	3α-Hydroxy-12-keto**	0.93
3α,6β-Dihydroxy**	0.55	3α,7α,12α-Trihydroxy (cholic)*	1.00
3-Keto-6α,7α-dihydroxy	0.55	3α,7α-Dihydroxy (chenodeoxycholic)*	2.17
3-Keto-7β-hydroxy	0.60	3α,12α-Dihydroxy (deoxycholic)*	2.41
3α,6α,7α-Trihydroxy (hyocholic)**	0.64	3-Keto***	4.79
3,12-Diketo**	0.64	3α-Hydroxy (lithocholic)*	5.89
3α,7β-Dihydroxy (ursodeoxycholic)*	0.66		

* Calbiochem.
** Steraloids.
*** Sigma.

TABLE II

RELATIVE CAPACITY FACTORS (*rk'*; RELATIVE TO CHOLIC ACID) AND SELECTIVITY (α) OF 3-KETO BILE ACIDS WITH THEIR CORRESPONDING α-HYDROXYL-SUBSTITUTED 5β-CHOLAN-24-OIC BILE ACIDS

Prefixes tauro- and glyco- refer to bile acids having taurine or glycine in amide linkage at C-24.

Hydroxyl/keto substituents and nature of conjugation	*rk'*	α
Tauro-3-keto-7α,12α-dihydroxy*	0.192	1.672
Tauro-3α,7α,12α-trihydroxy (taurocholic)*	0.321	
Glyco-3-keto-6α-hydroxy	0.210	1.286
Glyco-3α,6α-dihydroxy (glycohyodeoxycholic)**	0.270	
Glyco-3-keto-7α,12α-dihydroxy	0.253	1.672
Glyco-3α,7α,12α-trihydroxy (glycocholic)*	0.423	
Tauro-3-keto-7α-hydroxy	0.372	1.621
Tauro-3α,7α-dihydroxy (taurochenodeoxycholic)***	0.603	
Tauro-3-keto-12α-hydroxy	0.423	1.610
Tauro-3α,12α-dihydroxy (taurodeoxycholic)*	0.681	
3-Keto-7α,12α-dihydroxy*	0.591	1.692
3α,7α,12α-Trihydroxy (cholic)*	1.000	
Tauro-3-keto	0.293	1.332
Tauro-3α-hydroxy (taurolithocholic)*	1.229	

* Calbiochem.
** Steraloids.
*** Sigma.

TABLE III

RELATIVE CAPACITY FACTORS (*rk'*; RELATIVE TO GLYCOCHOLIC ACID) AND SELECTIVITY (α) OF 7-KETO BILE ACIDS WITH THEIR CORRESPONDING α-HYDROXYL-SUBSTITUTED 5β-CHOLAN-24-OIC BILE ACIDS

Prefixes tauro- and glyco- refer to bile acids having taurine or glycine in amide linkage at C-24.

Hydroxyl/keto substituents and nature of conjugation	*rk'*	α
Glyco-3α,12α-dihydroxy-7-keto	0.425	2.353
Glyco-3α,7α,12α-trihydroxy (glycocholic)*	1.000	
Tauro-3α-hydroxy-7-keto	0.578	2.407
Tauro-3α,7α-dihydroxy (taurochenodeoxycholate)***	1.391	
Glyco-3α-hydroxy-7-keto	0.743	2.392
Glyco-3α,7α-dihydroxy (glycochenodeoxycholate)***	1.777	
3α,6α-Dihydroxy-7-keto	0.905	1.740
3α,6α,7α-Trihydroxy (hyocholic)**	1.575	

* Calbiochem.
** Steraloids.
*** Sigma.

module. Relative capacity factors (*rk'*) were determined at a flow-rate of 2.0 ml min^{-1} using a Radial-Pak C_{18}, 10 μm (8 mm I.D.) reversed-phase cartridge and a mobile phase of 100 ml of methanol–water (72.5:27.5) and 2.5 ml of glacial acetic acid with the final pH adjusted to 5.25 with 10 *M* sodium hydroxide. The Radial-Pak C_{18} cartridge has a void volume of 2.75 ml. Peak retention times were determined with an on-line computer and chromatography software (Trilab II, Trivector Scientific, Sandy, U.K.). Elution volumes were determined from the retention times and the flow-rate. Data collection was automatically instigated via the sample injector.

Chromatographic parameters

(a) Relative capacity factor, rk', $= \frac{k'_x}{k'_s} = \frac{V_x - V_0}{V_s - V_0}$ where rk' = relative capacity factor, V_0 = void volume, V_x = elution volume of bile acid of interest, V_s = elution volume of reference standard.

(b) Selectivity, α, $= \frac{rk'_a}{rk'_b}$ where a = the more retained bile acid and b = the less retained bile acid.

RESULTS AND DISCUSSION

The elution pattern of the keto bile acids studied was similar to that of the common bile acids[3] and generally eluted in the order taurine conjugates, glycine conjugates, free acids, and tri, di, mono substitution. The presence of a single keto group at C-3, C-7 or C-12 of the 5β-cholan-24-oic acids with hydroxyl groups only in the α position resulted in lower relative capacity factors than the corresponding hydroxylated compounds and eluted in the order C-7, C-12, C-3 substitution (Tables I–III). However, bile acids with hydroxyl-group substitution in the β configuration

at C-6 or C-7 gave lower relative capacity factors than the corresponding keto substitution. The presence of more than one keto group in the structure reduced the relative capacity factors compared to the mono keto substituents.

In general the relationship between bile acid structure and elution order is similar to that observed by Shaw *et al.*[5] using a stainless steel μBondapak C_{18} column and a propanol–phosphate buffer (pH 7.0) mobile phase. In both studies 6α-, 6β- and/or 7β-hydroxyl substitution resulted in retention volumes which could not be predicted merely on the basis of degree of hydroxylation. Apart from compounds with 6α-hydroxyl or substitution only at C-3, the relationship between α-hydroxylated bile acids differing in only a single keto or hydroxyl substituent appears to result in fairly uniform selectivity values (Tables II and III). Values in the range 1.61–1.69 were found for 3-keto substitution of tri- and di-hydroxy bile acids, while values of approximately 2.4 were observed for the corresponding 7-keto substitution.

Although no attempt was made to separate the complex mixtures of bile acids reported, the information given on relative capacity factors of keto- and hydroxyl-substituted bile acids will be of interest in the analysis of biological samples and to workers developing similar HPLC separation systems.

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