Radioimmunoassay for carbenoxolone

B. M. PESKAR, B. A. PESKAR*‡, J. C. TURNER†, *Medizinische Klinik, University of Freiburg, *Department of Pharmacology, University of Freiburg, D-78 Freiburg i. Br., West Germany and †Biorex Laboratories Ltd., London, H1 2HB, U.K.*

Carbenoxolone sodium (3-*O*-(β-carboxypropionyl)-11-oxo-18β-olean-12-en-30-oic acid disodium salt) is widely used in the treatment of gastric and duodenal ulcers. It can be determined in biological fluids using spectrophotometric (Coleman & Parke, 1963) or gas chromatographic (Rhodes & Wright, 1974) methods. We describe the production of antisera against carbenoxolone, their specificity and their use for a radioimmunoassay.

[^{3}H]Carbenoxolone (specific activity 4·5 mCi mg^{-1}), unlabelled carbenoxolone sodium and the carbenoxolone analogues were from Biorex Laboratories Ltd (London, U.K.). [^{3}H]Carbenoxolone was synthesized by reduction of 3-keto-enoxolone with sodium borotritiide followed by succinoylation of the resultant [^{3}H]-enoxolone with succinic anhydride. For preparation of the antigen carbenoxolone was conjugated to bovine serum albumin using the carbodiimide method described by Goodfriend, Levine & Fasman (1964). Bovine serum albumin (5 mg) (Calbiochem, San Diego, U.S.A.) was dissolved in 1 ml of distilled water. Then 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Ott Chemical Co. Muskegon, Mich., U.S.A.) and (5 mg) carbenoxolone sodium were added and the solution stirred at room temperature (20°) for 1 h and incubated over night. The pH of the reaction mixture was about 7·5. The resulting turbid conjugate was dialysed exhaustively for 5 days against 7 changes of distilled water (2 litres each). The conjugate was analysed spectrophotometrically and compared with a BSA-carbodiimide conjugate not containing carbenoxolone. The amount of carbenoxolone coupled to the BSA was calculated from the difference spectra and was found to be 10 moles per mole of BSA. An 0·3 ml aliquot of the conjugate (2·8 mg ml^{-1} in terms of bovine serum albumin) was diluted to 1·6 ml with 0·15 M NaCl and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, Mich., U.S.A.) and 0·25 ml of the emulsion was injected into each foot pad of three rabbits (about 2 kg) resulting in a total dose of 250 μg antigen (in terms of bovine serum albumin) per rabbit. Booster injections with half the amount of antigen were administered by the same route at weeks 1, 4, 6, 10 and 15. Blood was collected by puncture of the ear artery before immunization and 10–14 days after each antigen injection. The blood was allowed to clot at 4° overnight, the serum was separated by centrifugation at 2000 *g* for 15 min and the sera stored at —20°.

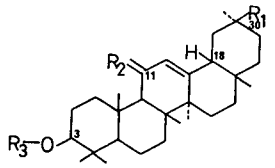
‡ Correspondence.

For titration experiments pre-immunization sera or antisera were diluted in standard diluent buffer (0·15 M NaCl —0·01 M sodium phosphate buffer, pH 7·8 containing 1 mg ml^{-1} gelatin). 0·1 ml of various serum dilutions were added to 1·9 ml of [^{3}H]carbenoxolone (about 3000 counts min^{-1} = 1·7 ng) in standard diluent buffer. The mixtures were incubated at 4° overnight. Then 2 ml of a charcoal suspension (20 mg ml^{-1}) was added to separate the free and antibody-bound fractions (Herbert, Lau & others, 1965). However, no dextran had been added to the charcoal. The samples were centrifuged immediately at 4° for 10 min (2000 *g*) and the supernatants added to 14 ml Scintigel (Roth, Karlsruhe, West Germany) and the radioactivity was measured. For radioimmunoassay of carbenoxolone the same protocol as in the titration experiments was used except that various amounts of unlabelled carbenoxolone were added in a volume of 0·1 ml and the [^{3}H]carbenoxolone was added in a volume of 1·8 ml. For these experiments a final antiserum dilution, which could bind about 50% of the label (1:3000), was used. Control tubes with the same dilution of pre-immunization serum were included in all assays. For determination of specificity of the antisera carbenoxolone was replaced by various carbenoxolone analogues (Table 1). While carbenoxolone sodium, 18α-carbenoxolone sodium, ammonium glycyrrhizinate, BX 441, BX 494 and BX 363 A are water soluble at the pH used, enoxolone, BX 310 and BX 311 were first dissolved in ethanol (10 μg ml^{-1}). They were then further diluted in standard diluent buffer containing 1% ethanol and added to the incubation mixtures. The presence of 1% ethanol in the incubation mixtures did not interfere with the radioimmunoassay.

All three rabbits immunized with the carbenoxolone-protein conjugate produced antibodies against carbenoxolone. A final antiserum dilution of 1:3000 can bind 57% of the added [^{3}H]carbenoxolone. On the other hand under the incubation conditions used serum obtained from the same rabbit before immunization even in the highest concentration used (1:250) did not bind more then 3% of the added [^{3}H]carbenoxolone.

The sensitivity of the radioimmunoassay for carbenoxolone allows the measurement of 1 ng carbenoxolone. Since the inhibitor can be added to the radioimmunoassay in volumes up to 1 ml, 1 ng ml^{-1} carbenoxolone can be detected by this method. The standard curve for carbenoxolone is linear from 1·2 up to almost 50 ng, 5·5 ng causing 50% displacement of the labelled antigen from the antiserum (Table 1). The specificity of the antiserum is illustrated in Table 1. While the antiserum

Table 1. *Structure of carbenoxolone and carbenoxolone analogues used for determination of specificity of anti-carbenoxolone antiserum and their interference in the carbenoxolone radioimmunoassay.*



Compounds	R_1	R_2	R_3	50% displacement of ^{3}H-carbenoxolone (ng)
Carbenoxolone sodium	CO_2Na	O	$CO{\cdot}[CH_2]_2{\cdot}CO_2Na$	5·5
BX 310	CO_2Na	O	$CO{\cdot}[CH_2]_3{\cdot}CO_2Na$	6·0
Enoxolone	CO_2H	O	H	6·0
BX 363 A	CO_2Na	O	$CO{\cdot}CH{\cdot}[CH_2]_4CH{\cdot}CO_2Na$	34·0
18α-Carbenoxolone sodium*	CO_2Na	O	$CO{\cdot}[CH_2]_2{\cdot}CO_2Na$	55·0
Ammonium glycyrrhizinate	CO_2H	O	$GlcA[NH_4]{\cdot}GlcA$	100·0
BX 441	CO_2Na	H_2	$CO[CH_2]_2{\cdot}CO_2Na$	>100·0
BX 311	$CO_2{\cdot}CH_2{\cdot}CH{:}CH{\cdot}C_6H_5$	O	$CO{\cdot}CH_3$	>100·0
BX 494 *†	CO_2Na	O	H	>100·0

* Substitution at C_{18} in α-position;
† Substitution at C_2: $-CH_2{\cdot}O{\cdot}CO[CH_2]_3{\cdot}CO_2Na$.

cannot distinguish clearly between carbenoxolone and analogues with moderate changes in the substitutions at C_3 like BX 310 or even the hydrolysis product enoxolone, molecules with larger substitutions at C_3 like BX 363 A and ammonium glycyrrhizinate or an additional substitution at C_2 like BX 494 interfere less with the radioimmunoassay. Relatively little interference is seen with BX 311, an analogue with a large substitution at C_{30} and BX 441 differing from carbenoxolone by the missing keto group at C_{11}. Surprisingly little interference is seen with 18α-carbenoxolone (10% cross-reaction). These results indicate that the antisera are relatively most specific for the substitutions at positions C_{11}, C_{18} an C_{30} of the carbenoxolone molecule, while greater changes at the C_3 position are necessary to be recognized by the antisera. Two explanations are possible for these results. From the work of Beiser, Erlanger & others (1959) on the antigenicity of steroid-conjugates it is known that the antibodies are most specific for those parts of the hapten, which are remote from the point of attachment to the carrier protein. If most of the carbenoxolone molecules had been coupled to the bovine serum albumin through the C_3-side chain, this phenomenon could explain the relative lack of specificity of the antibodies against the determinants at this position. Alternatively some of the carbenoxolone could have been hydrolysed during the preparation of the antigen or after immunization of the rabbits, resulting in antibody populations directed predominantly against enoxolone. The [^{3}H]carbenoxolone used in the radioimmunoassay would then be bound by less specific cross-reacting antibodies. Only large substitutions at C_3 would then significantly impair the chances of a molecule to displace [^{3}H]carbenoxolone from the antibodies.

While hydrolysis of carbenoxolone to enoxolone and succinate occurs extensively in the rat, it occurs in man only to a small extent (Johnston, Lindup & others, 1974). Therefore, the radioimmunoassay for carbenoxolone should be suitable for pharmacokinetic studies in man. The radioimmunoassay is presently being used to measure plasma and tissue levels of carbenoxolone.

The validity of the radioimmunoassay method has been checked by comparison with the existing g.l.c. method of Rhodes & Wright (1974). Three serum samples containing 13·4, 97·7 and 40·6 μg ml^{-1} carbenoxolone respectively were analysed. The results with the g.l.c. method were 14, 99 and 41 μg ml^{-1}, while with the radioimmunoassay method 11·3, 92·0 and 35·8 μg ml^{-1} were measured.

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