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A gas chromatographic determination of carbenoxolone in human serum

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A gas-liquid chromatography procedure for the determination of carbenoxolone in human serum is described. The method showed a better than ± 11% coefficient of variation for all values determined. The mean determined value is accurate to ± 10% of the known value for carbenoxolone over a range of 5 to 100 μg ml^{-1} serum. The specificity of the method was established by combining the gas-liquid chromatography analysis with thin-layer chromatography of prepared standards and serum from treated volunteers.The method is suitable for routine pharmacokinetic and bioavailability studies of carbenoxolone in serum and other biological fluids and supersedes previous methods in specificity, sensitivity and simplicity of determination.

Carbenoxolone sodium (3-*O*-(β-carboxypropionyl)-11-oxo-18β-olean-12-en-30-oic acid disodium) is used in the treatment of gastric and duodenal ulcers (Doll, Hill & others, 1962; Doll, Hill & Hutton, 1965; Craig, Hunt & others, 1967; Robson & Sullivan, 1967; Baron & Sullivan, 1970). However, the determination of its pharmacokinetics in man has been limited by the lack of a suitable method for its assay in serum and other biological materials.

Although a method for measuring carbenoxolone in biological fluids (Coleman & Parke, 1963) and studies on its absorption in man (Downer, Galloway & others, 1970; Parke, Hunt & Iveson, 1972; Lindup, Parke & Colin-Jones, 1970) have been described, no suitable method for routine application to man has so far been developed.

A gas chromatographic determination of carbenoxolone has now been devised following a simple extraction of the drug from serum. The method is convenient, accurate and specific for the determination of carbenoxolone in serum and other biological fluids. The technique can be adapted to the determination of other metabolites of carbenoxolone as well as other triterpenoid-type drugs.

MATERIALS AND METHODS

Materials

Carbenoxolone (β-glycyrrhetic acid hydrogen succinate), (3-*O*-β-carboxypropionyl-11-oxo-18-β-olean-12-en-30-oic acid), m.p. 319°, $[\alpha]_D^{20}$ + 131° in 1% chloroform and the corresponding disodium salt that had been dried over phosphorous pentoxide for 18 h under vacuum at 130°; the corresponding 18α-isomer (3-*O*-(β-carboxypropionyl)-11-oxo-18-α-olean-12-en-30-oic acid), m.p. 302–303°, $[\alpha]_D^{20}$ + 80° (1% in chloroform) and its disodium salt dried as previously described, were all prepared at Biorex Laboratories. Prepared silica gel HF 254 plates were obtained from Anderman & Co. All other solvents and reagents were Analar grade (BDH).

Methods

A Pye Unicam series 104 gas chromatograph fitted with a flame ionization detector and injector point heater was employed. Chromatography of methylated derivatives was performed on 1′ × ¼″ glass columns containing 1% OV-1 (dimethyl silicone gum) on a solid support of Gas-Chrom Q (100-120 mesh) (Pye Unicam, England) at a temperature of 265°. All gas settings were optimum for the instrument.

The 18α-isomer of carbenoxolone was employed as the internal standard and results were quantitated by a comparison of peak heights.

An ethereal solution of diazomethane prepared *in situ* from *p*-toluene sulphonyl methyl nitrosamide (Fieser & Fieser, 1967) was used to prepare methyl derivatives of the extracted carbenoxolone before chromatography.

Preparation of standard solutions. All standard solutions were prepared in absolute ethanol and stored, stoppered, at + 4°. Test solutions containing sodium carbenoxolone were prepared by evaporating to dryness known volumes of standard ethanol solution adding known volumes of serum or water and mixing in a Vortex mixer (Griffin & George). Analysis was performed on 1 ml aliquots of the test solutions.

Standard procedure. A known amount of the standard disodium 18α-carbenoxolone solution, acting as internal and recovery standard, was added to 1 ml of test sample contained in a 15 ml stoppered tube. The solution was acidified with 4 ml of 1M HCl and extracted twice with 5 ml of ether using a rotary-inversion mixer (15 min). After centrifugation (500 *g*; 5 min) each ether layer was transferred to a stoppered tube and the ether evaporated on a water bath. The residue was redissolved in ethanol (0·5 ml) and 0·5M Na_2CO_3 (4 ml) added. The alkaline solution was extracted twice with ethyl acetate (5 ml) and the aqueous layer separated, acidified with 2·5M HCl and extracted twice with ether (5 ml) as previously described. The ether layers were transferred to a tapered tube, reduced in volume, methylated with an ethereal solution of diazomethane and reduced to dryness. The methylated residues were dissolved in ethyl acetate (200 μl) and an aliquot injected onto the 1% OV-1 column at 265° for g.l.c. analysis.

To increase the sensitivity of the assay, a smaller volume of ethyl acetate may be used to solubilize the methyl derivatives. A standard of β-carbenoxolone, in triplicate, was included in each series of analyses as a check on the internal standard.

RESULTS AND DISCUSSION

Direct gas chromatography of ether extracts from serum revealed two impurities that gave identical elution times when chromatographed before and after methylation with diazomethane on 1% OV-I stationary phase. Both serum components (I & II) showed differing retention times to the α- and β-carbenoxolone peaks (Fig.1).

It was not possible when using OV-1 stationary phase to eliminate the preliminary purification steps, as the quantities of serum impurity in non-purified extracts was such that they interfered with the quantitation of the carbenoxolone peaks.

Specificity of assay

To establish the specificity of the assay for human serum both prepared standard serum samples (100 μg β-carbenoxolone per ml) and serum samples obtained from volunteers receiving carbenoxolone sodium (Biogastrone) were subjected to various

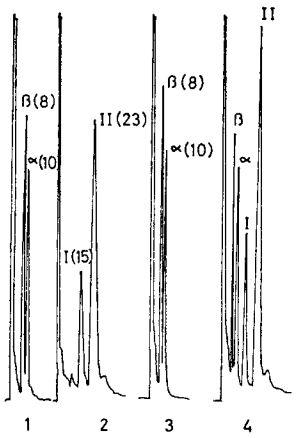

FIG. 1. Gas chromatograms of β-carbenoxolone (β), α-carbenoxolone (α) and serum impurities (I and II) on 1% OV-1. (1) Standard solution of β- and α-carbenoxolone dimethyl derivatives. (2) Impurities from serum diluted 100 × relative to corresponding carbenoxolone solutions. (3) Standards of β- and α-carbenoxolone sodium extracted from serum showing elimination of serum impurities by back extraction technique, chromatographed as methyl derivatives. (4) Standards of β- and α- carbenoxolone dimethyl derivatives added to serum impurities to show no coincidence of peaks. Figures in brackets show retention time of peaks in min.

modifications of the standard procedure. Serum samples were analysed by the standard procedure using chromatography on 1% OV-1. The same serum samples after methylation were subjected to chromatography on thin-layer plates (0·25 mm) of silica gel HF254 developed in the solvent system chloroform–ether (3:1 v/v). The spots corresponding to the methylated carbenoxolone standards were extracted with ethyl acetate (2 × 3 ml) and after concentration the carbenoxolone content was determined by gas chromatography on 1% OV-1 (Table 1). In other serum samples, the extracted free acids before methylation were chromatographed on thin-layer plates (0·25 mm) of silica gel HF254 developed in the solvent butanol–aqueous

Table 1. *A comparison of serum carbenoxolone determinations after thin-layer chromatography (t.l.c.) combined with the standard gas-liquid chromatography (g.l.c.) procedure.*

	Carbenoxolone concn determined (μg ml^{-1})		
	Standard method g.l.c. OV-1	t.l.c. of free acid g.l.c. OV-1	t.l.c. of methylated derivative g.l.c. OV-1
Carbenoxolone concn in prepared serum samples (100 mg ml^{-1})	91	100	
	109	127	
	105		103
	95		99
	100		99
Carbenoxolone in serum from volunteers	43	45	
	43	45	
	145	168	
	126	145	
	34		35
	50		62
	15		15
	29		27
	35		31

ammonia solution (s.g. 0·88) (5:1 v/v) and the spots corresponding to the carbenoxolone standards were extracted with methanol (2× 3 ml). The extracts were methylated and concentrated and subjected to gas chromatography on 1% OV-1.

Accuracy of the standard procedure

The ratio for the peak heights of the methyl derivatives of α- and β-carbenoxolone was determined for a series of standard solutions chromatographed directly on 1% OV-1. This was compared with the same standard solutions that had been processed through the extraction procedure. No significant differences ($P>0·01$) were found between the ratio of extracted standards (1·15 ± s.e. 0·01, n = 6) and those chromatographed directly (1·15 ± s.e. 0·04, n = 6). It is recommended, however, that a standard carbenoxolone solution should be included during each analysis when internal standard solutions that have not been freshly prepared are used.

The ratio of the peak heights of the methyl esters of β-carbenoxolone and α-carbenoxolone determined for a single standard sample containing 100 μg of β-carbenoxolone, analysed 25 times employing the standard extraction procedure and the ratio against an internal standard of α-carbenoxolone (100 μg ml^{-1}), was found to be 1·15 ± s.e. 0·01 (n = 25), which established excellent precision for the analysis under these optimum conditions of internal standard concentrations.

The standard procedure was applied to human serum samples prepared as previously described. In all analyses, samples were randomized before the determination to eliminate operator bias. The results (Table 2) show that good correlation was obtained between the mean of the determined value and the expected value up to 100 μg ml^{-1} with an accuracy within ± 10%. The coefficient of variation was between 3·3 and 10·2% for the whole series of determinations indicating good reproducibility at both high and low concentrations of carbenoxolone.

Table 2. *Determination of carbenoxolone in prepared serum and water samples employing the standard procedure.*

	Internal standard concentration (μg ml^{-1})	Carbenoxolone concn (μg ml^{-1})	Mean determined carbenoxolone concn (μg ml^{-1}) ± s.d.
a) Serum	10	10	9·8(6)* ± 0·5
		100	98·5(6) ± 3·3
	20	10	10·5(4) ± 0·6
		20	20·0(4) ± 2·0
		30	34·0(4) ± 4·0
		40	42·6(4) ± 3·6
	50	5	5·1(9) ± 0·4
		50	47·8(9) ± 2·0
		150	134·0(10) ± 8·0
	100	10	10·6(6) ± 0·6
		100	98·0(6) ± 2·3
(b) Water	10	10	10·0(6) ± 0·1
		100	104·6(4) ± 6·8
	100	100	100·2(6) ± 0·1

* Number of determinations.

A series of blanks were also analysed during each of the analyses shown in Table 2 and no false carbenoxolone peaks were detected that were greater than 0·5 μg ml^{-1}.

A series of serum blanks from volunteers showed a similar pattern with no false positives above 0·5 μg ml^{-1}.

The method is capable of reliably estimating, with an accuracy of $\pm$ 10%, carbenoxolone at concentrations as low as 5 μg ml^{-1}. It is readily adaptable to estimate lower levels of carbenoxolone but the routine determination of the drug in human blood serum samples by this method has shown that this degree of sensitivity is unnecessary. The method is specific for carbenoxolone and false positive values have not been detected.

It is possible to adapt the method with minor modifications for the analysis of glycyrrhetic acid and preliminary studies indicate that it can also be applied to the analysis of carbenoxolone glucuronide or sulphate conjugate in bile after preliminary hydrolysis of the conjugates with β-glucuronidase and/or arylsulphatases.

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