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Note

Determination of natural cholestenone in rat adrenal extracts by high-performance liquid chromatography

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The conversion of 5-unsaturated-3β-alcohols into the corresponding 4-unsaturated-3-ketones is an important reaction in steroid metabolism and in steroid chemistry and represents the last step in the commercial synthesis of a variety of hormones. Also, the formation of cholest-4-en-3-one (cholestenone) *in vivo* involves the initial oxidation of cholesterol by 3-hydroxysteroid dehydrogenase, probably via cholest-5-en-3-one[1]. 5β-Cholestan-3β-ol (coprostanol), a major steroid found in faeces, is also produced by the oxidation of cholesterol via cholestenone[2]. Prolonged feeding of cholestenone to rats results in adrenal hypertrophy and suppression of cholesterol synthesis[3–5].

Thin-layer chromatography (TLC) has been used for the analysis of cholestenone with detection by iodine vapour, by radioautography[6], by examination of the chromatoplate under 254-nm UV light or by spraying with 50% aqueous sulphuric acid followed by gentle warming[7]. High-performance liquid chromatography (HPLC) has also been employed in order to separate the autooxidation products of cholesterol[8]. The effects of stereochemical changes at C(5) of C_{27} sterols on their adsorption were illustrated by the elution patterns of the corresponding 3-keto analogues[9].

The favourable characteristics of HPLC, *i.e.*, the simple preliminary treatment of the sample, the possibility of very selective separations and the high sensitivity, suggested its use for studying the mechanism of the action of cholestenone on the production and steroidogenesis of endogene corticosteroids in the rat, the aim being to evaluate the accumulation of cholestenone in the adrenal.

EXPERIMENTAL

Preparation of standard and sample

The chromatographic reference standard was prepared by dissolving 0.1 mg of cholestenone in 10 ml of ethanol. This solution is stable at 4°C for at least three months. The purity of the cholestenone was previously checked by HPLC (less than 2% area contribution from impurity peaks).

The adrenal was extracted by the method described by Folch *et al.*[10], one adrenal yielding 5 ml of extract. A part of the chloroform–methanol extract (1/5–1/10 of the whole) was evaporated under a stream of nitrogen, the residue dissolved in 100 μl of methanol and a part of this solution (10–30 μl) was injected into the column.

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Chromatography

The HPLC system consisted of a 2501 HP pump (Laboratory Instruments, Prague, Czechoslovakia), a low-volume inlet port with septum and a 100 × 4 mm column packed with Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.). The balanced-density slurry method was employed to pack the column. The mobile phase was methanol–dichloromethane–water (96:2:2) at a flow-rate of 0.9 ml/min. All the components of the mobile phase were redistilled on a glass column. The detection was carried out by a Perkin-Elmer LC-55B variable-wavelength UV–visible detector connected with an LC-55S digital scanner. The column effluent was monitored at 244 nm.

Animals

Male and female Wistar rats were used. They were normally fed a standard diet except for a group which were supplied for 7 days with doses of 0.25 g cholestenone per rat per day.

RESULTS AND DISCUSSION

Optimum conditions

Various chromatographic conditions were evaluated by injecting 100 ng of cholestenone in 10 μl of methanol. The variations in the mobile phase included the use of different methanol–dichloromethane–water ratios. The 96:2:2 mixture gave the desired separation in 7 min, with a retention time of 6 min for cholestenone. Fig. 1 illustrates a chromatogram of adrenal extracts for two different concentrations of cholestenone: (1) from an extract of the normal rat adrenal; (2) from the adrenal of a rat fed with cholestenone for 7 days. The maximum absorbance of cholestenone occurred at 244 nm when it was scanned in the mobile phase.

Evaluation of the method

Sensitivity. The limit of detection of cholestenone was 10 ng at a signal-to-noise ratio of 2.5 or greater.

Precision. To determine the precision, ten samples containing 100 ng of cholestenone were analyzed. With model samples prepared from the pure compound, the coefficient of variation was 1.6%, whereas with a pooled adrenal homogenate the coefficient of variation was 4%.

Linearity. The peak-height response was linear over the range 10–200 ng of cholestenone applied on the column.

Recovery. Known amounts of cholestenone dissolved in ethanol were added to a normal adrenal homogenate pool; the amounts shown in Table I were found when 10-μl charges of the extract were injected. The absolute recovery was determined by comparing the peak heights recorded upon injecting the extracts and pure cholestenone standards. The recovery of cholestenone was 95–108%.

Specificity. The specificity of the method is enhanced by the combined effects of solvent extraction, appropriate choice of wavelength and chromatographic separation. Bush[11] showed that corticosterone was the major product of rat adrenal tissue, rather than cortisol as in other species. Fig. 2 presents two chromatograms obtained by injecting: (1) 100 ng of cortisol and 20 ng of corticosterone; (2) directly 100 ng of

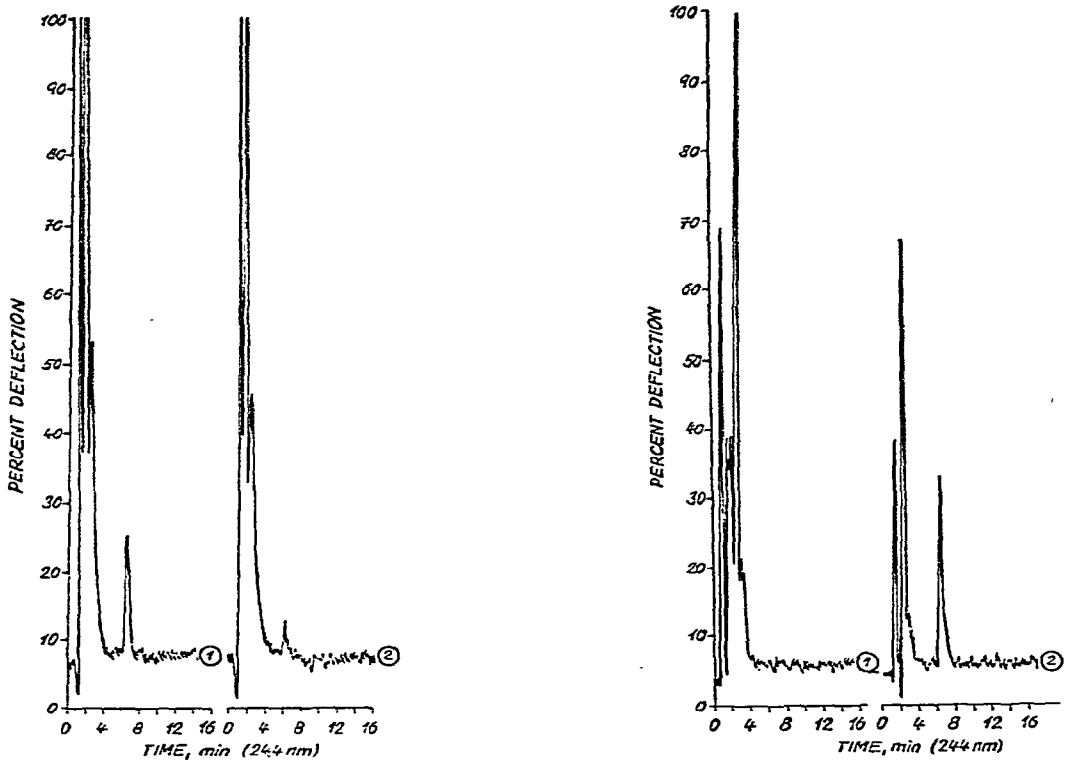

Fig. 1. Chromatograms of an extract of: (1) the normal rat adrenal; (2) the rat adrenal fed for 7 days with 0.25 g cholestenone per day.

Fig. 2. Chromatograms of: (1) 100 ng cortisol and 20 ng corticosterone; (2) 100 ng cholestenone.

cholestenone. As expected, cortisol and corticosterone are eluted very much earlier than cholestenone in the reversed-phase chromatographic system. The same results were obtained with progesterone.

The presence of steroids with polarities similar to that of cholestenone, *e.g.*, cholesta-3,5-diene, a dehydration product of cholesterol ($\lambda_{max.}$ 235 nm), or some other conjugated steroid dienes was excluded by the simple test of the reaction of cholestenone in rat adrenal extracts with 2,4-dinitrophenylhydrazine[12]. After the reaction the peak corresponding to cholestenone had disappeared.

TABLE I

ANALYTICAL RECOVERY OF CHOLESTENONE FROM AN ADRENAL HOMOGENATE

Amount of cholestenone [nmol (ng)] in a 10-µl charge		*Recovery (%)*
Added	*Recovered*	
0.648 (250)	0.700	108
1.27 (500)	1.31	102
2.59 (1000)	2.63	103
5.18 (2000)	5.44	95

Constant absorbance ratios of pure cholestenone and of that extracted from the rat adrenal, scanned over a wavelength range of 230–330 nm while stopping the flow, served as an additional measure of specificity.

Cholestenone levels. The mean absolute content of cholestenone, calculated from twenty normal rat adrenals, was 2.26 μg, the mean weight of one adrenal being 27.66 mg. The limiting values were 1.0 and 4.2 μg of cholestenone and 24.0 and 30.7 mg for one rat adrenal. The mean cholestenone concentration was 81 μg/g, the limiting values being 32 and 165 μg/g. There are no literature reports on the levels of cholestenone in normal rat adrenal.

Applicability. The extraction procedure does not differ from that used in TLC. The time necessary to carry out the chromatographic separation of one sample is about 7 min, the consumption of the mobile phase being about 6.5 ml. The method has proved reliable. We have also used this separation technique, without any changes, for the estimation of cholestenone in the rat testes.

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