Gas–liquid chromatography of steroid glucuronosides

Among the biologically important steroids amenable to gas–liquid chromatographic (GLC) analysis are the 17-ketosteroids, estrogens, testosterone and pregnanediol[1,2]. Certain of these compounds, the urinary levels of which reflect endocrine activity, are conjugated with glucuronic acid prior to their excretion. Methods for the determination of these steroids in urine usually call for chemical or enzymic hydrolysis of the conjugates to the free steroids which may then be determined by GLC, often as the trimethylsilyl (TMSi) ethers[1,2]. Although the size and complexity of the conjugated steroids themselves might be thought to preclude their direct separation by GLC, other types of compounds which appeared to present insurmountable challenges to this technique have been chromatographed successfully. For example, triglycerides of from 50 to 62 fatty acid carbon atoms have been separated[3], and a tetrasaccharide transformed to its fully trimethylsilylated ether (molecular weight 1676) has been eluted from a GLC column[4]. What may appear to be an excessively high molecular weight will not necessarily lead to an impractically long retention time, since it is the free energy of solution, rather than molecular weight, which determines retention behavior. Further, through the use of derivative formation compounds which possess exceedingly low vapor pressures may be converted to more volatile substances.

With this as a background, and using androstan-17-one-3α-yl-β-D-glucopyranosiduronic acid and 5-androstene-17-on-3β-yl-β-D-glucopyranosiduronic acid as model compounds, an investigation was carried out to determine the feasibility of the GLC of steroid glucuronosides.

Methylation of the carboxyl group (diazomethane in methanol–ether) was followed by trimethylsilylation of the hydroxyl groups of the sugar portion of the molecule by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine[4]. The GLC behavior of the reaction products and several reference standards is presented in Table I. The steroid glucuronoside derivatives are retained much longer than their parent steroids with the non-polar stationary phase SE-30 at 250°, a

TABLE I

Compound	*Relative retention time*[a]	
	SE-30	*NGS*
Androstan-3α-ol-17-one	1.00	1.00
Androstan-3α-ol-17-one-TMSi	0.94	0.22
Androstan-17-one-3α-yl-β-D-glucopyranosiduronic acid methyl ester tri-trimethylsilyl ether[b]	15.8	4.72
5-Androsten-3β-ol-17-one	1.00	1.18
5-Androsten-3β-ol-17-one-TMSi	1.15	0.34
5-Androsten-17-one-3β-yl-β-D-glucopyranosiduronic acid methyl ester tri-trimethylsilyl ether[c]	29.0	11.0

[a] Experimental conditions: 1.5% SE-30 on 100-120 mesh Gas-Chrom P; 6 ft. × 4 mm glass U-tube; 250°; 18 p.s.i.; absolute retention time of androstan-3α-ol-17-one, 1.6 min. 2% NGS on 80-100 mesh Gas-Chrom P; 3 ft. × 4 mm glass U-tube; 240°; 11 p.s.i.; absolute retention time of androstan-3α-ol-17-one, 2.7 min.

[b] The parent glucuronoside was purchased from Mann Research Laboratories.

[c] The parent glucuronoside was generously provided by the Medical Research Council (Great Britain) through the Steroid Reference Collection Office of the National Institutes of Health.

reflection of the great differences in molecular weight. The volatility difference between a derivatized steroid glucuronoside and its parent steroid is smaller with the polyester neopentylglycol succinate (NGS) at 240° than with SE-30, probably because the presence of a polar hydroxyl group causes the steroid to interact strongly with the polar phase. Etherification would mask this group and reduce the polarity of the steroid. When such a reduction in polarity is effected by trimethylsilyl ether formation with androstan-3α-ol-17-one and 5-androsten-3β-ol-17-one, large increases in volatility are observed with NGS (Table I). Even with SE-30, a stationary phase general y noted for separations based upon molecular weight[1], the TMSi ether of androstan-3α-ol-17-one is actually eluted faster than the lower molecular weight parent steroid, and trimethylsilylation of 5-androsten-3β-ol-17-one does not lead to a large increase in retention time (see Table I). Indeed, trimethylsilylation results in a much smaller increase in retention time on non-polar phases than formation of butyryl esters, although the associated molecular weight changes are rather similar[1]. It is clear that the TMSi group possesses somewhat anomalous volatility properties. The glucuronosides may be considered steroids etherified by glucuronic acid at the 3-position (like TMSi ethers but with a much larger functional group), and the effect upon volatility of the introduction of such a large function into a steroid is difficult to predict. What is clear, however, is that the derivatized glucuronosides can be chromatographed under conditions which are not radically different from those normally employed in steroid GLC. The separation of the methyl ester tri-trimethylsilyl ether derivatives of the β-D-glucopyranosiduronic acids of androstan-3α-ol-17-one and 5-androsten-3β-ol-17-one with SE-30 is illustrated in Fig. 1, and the theoretical shape of the peaks indicates the excellent GLC properties of these compounds*.

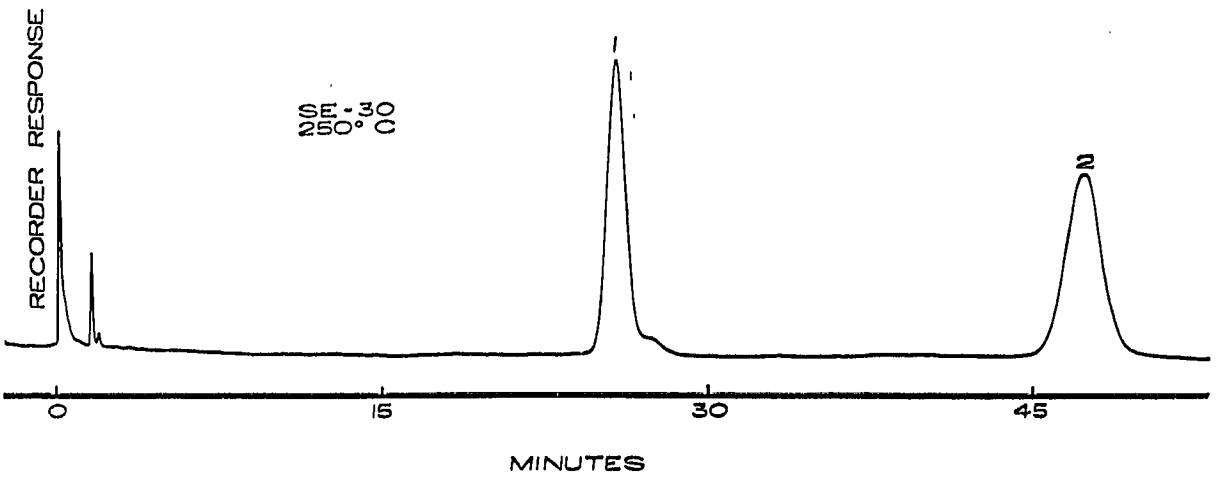

Fig. 1. Gas–liquid chromatographic separation of a mixture of the methyl ester tri-trimethylsilyl ethers of the β-D-glucopyranosiduronic acids of androstan-3α-ol-17-one (peak 1) and 5-androsten-3β-ol-17-one (peak 2). Column conditions given in Table I.

Although the two derivatized steroid glucuronosides differ only by a double bond and the stereochemistry of the substituent group at the 3-position, their retention

* When the products from esterification of the steroid conjugates were analyzed on SE-30 in each case only a small peak of non-theoretical shape was observed (shorter retention times than the fully derivatized compounds). GLC of equivalent amounts of the trimethylsilylated esters resulted in much larger symmetrical peaks.

behavior is widely different. With both stationary phases the derivatized glucuronoside of 5-androsten-3β-ol-17-one is retained much longer than the derivatized glucuronoside of androstan-3α-ol-17-one (see Table I and Fig. 1), and this order of elution is not unexpected. Separation of closely related steroids, especially those differing in the configuration of a hydroxyl group at the 3-position, is generally increased by derivative formation (the axial isomer exhibiting a shorter retention time than the equatorial), the separation factor increasing with the steric requirements of the substituent group[1]. This effect can be seen in Table I, where with SE-30 the parent steroids possess the same retention times, but after trimethylsilylation a large difference in volatility is noted. NGS, a phase selective for equatorial hydroxyl groups and double bonds, retains 5-androsten-3β-ol-17-one longer than androstan-3α-ol-17-one, but the effect is considerably increased by TMSi ether formation, although this separation factor and the corresponding one with SE-30 are much smaller than those observed for the derivatized glucuronosides.

Because of the paucity of the two steroid glucuronosides available to us it was not possible to obtain classical elementary analyses of their derivatives. The GLC behavior observed for these two compounds, however, is consistent with their possessing the assigned structures, and the data obtained by combined GLC–mass spectrometry[5,6] support the proposed chemical nature of the derivatives.

The application of the herein described separation technique to the identification and estimation of steroid glucuronosides in urine was beyond the scope of this investigation*. The essence of this communication is a further demonstration that experimental conditions may be established for the successful GLC of natural products of considerable structural complexity.

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* Work of this nature is presently being carried out, however (I. JAAKONMÄKI and E. C. HORNING, personal communication).