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LIPOPHILIC GEL AND GAS-PHASE ANALYSIS OF STEROID HORMONES

APPLICATION TO THE HUMAN NEWBORN

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SUMMARY

Steroid extracts from the urine of newborn infants can be separated into two fractions according to their mode of conjugation; metabolites excreted in the sulphate and glucuronide fractions appeared to be of different biological significance. Both fractions still represent complex mixtures of structurally related compounds which are not completely resolved by gas–liquid chromatography (GLC). Methods are presented for the fractionation of the extracts by lipophilic gel chromatography on a highly substituted hydroxyalkoxypropyl Sephadex derivative. A straight-phase system eluted with benzene permits the separation of groups of steroids according to the number of hydroxyl groups in the molecule. A reversed-phase system eluted with methanol–*n*-heptane (9:1) permits the rapid isolation of the polar steroids. Both systems provide additional structural information of the chromatographed steroids and form the basis of useful separation and purification procedures prior to GLC and gas chromatography–mass spectrometry (GC–MS). An example is given of the application of the straight-phase system to the fractionation of a urinary extract for GC–MS and mass fragmentography.

INTRODUCTION

The urine of human newborn infants has been shown to contain large amounts of steroid metabolites which are undetectable in later life, the most typical compounds being $\Delta 5,3\beta$-hydroxylated structures, excreted as sulphates[1–3]. A biological significance remains to be attributed to these compounds during perinatal life, if they are not considered simply as a persistance of foetal biosynthetic activity in the liver and adrenal of newborn infants. A number of metabolites are still of unknown structure. Their identification should point to the quantitatively important enzymatic pathways, some of which might be of interest as possible markers of differentiation of liver steroid metabolism as shown in the rat for the case of the 15α-hydroxylase system[4].

Progress in this field has been dependent on available methodology. Steroid

metabolites in extracts of such biological fluids as urine or plasma represent complex mixtures of structurally related compounds and, in addition, non-steroidal lipids are normally present which often lead to interference in the analysis.

Purification methods have included paper, thin-layer[5–7] and silicic acid column chromatography[8]. Gas–liquid chromatography (GLC) and gas chromatography–mass spectrometry (GC–MS) have been used either for the study of purified individual compounds[7,8] or as an approach to steroid metabolic profiles[9,10]. The resolution and structural information given by GLC have been improved by the introduction of new types of derivatives[11,12] and by the development of suitable open tubular glass capillary columns [13,14].

For the preliminary steps of purification, liquid column chromatography on lipophilic dextran gels is particularly suitable with respect to reproducibility, sample recovery and chemical activity[15,16]. The commercially available gel Sephadex LH-20 has been successfully applied to the group separation of urinary and plasma steroids prior to GLC, GC–MS or other techniques of measurement[17–19].

In the present work, we have examined lipophilic gel chromatography using a highly substituted derivative of Sephadex G-25 as a pre-step to GLC and GC–MS characterization of neutral urinary steroids in newborn infants. The aim was to simplify the complex mixtures to be subjected to GLC separation and simultaneously to obtain a further parameter of structural information for the individual components under study. In addition, fractions with minimal contamination are necessary if mass fragmentography is to be carried out with biological extracts.

Several novel reference compounds have been synthesized to help in the identification and isolation of the corresponding steroids in urinary extracts.

EXPERIMENTAL

Preparation of urinary extracts

Samples of 24-h urine (average volume 28.5 ml) were collected from normal human newborns aged 1–5 days and kept frozen at −25° until processed.

Total steroid extracts were obtained after enzymatic hydrolysis of urine (0.2 *M* acetate buffer, pH 5.2) with *Helix pomatia* (Industrie Biologique Française, Gennevilliers, France) for 48 h at 37°. The hydrolyzed urine was extracted with two volumes of diethyl ether and two volumes of ethyl acetate. The combined organic phase was washed with 0.1 volume of 0.1 *N* sodium hydroxide solution and distilled water, filtered through anhydrous sodium sulphate and evaporated to dryness prior to derivative formation and/or gel chromatography.

Steroid sulphate and glucosiduronate fractions were obtained by a two-step differential hydrolysis[20]. The urine was brought to pH 1 (sulphuric acid); after addition of sodium chloride (20%, w/v), it was extracted with two volumes of ethyl acetate. Solvolysis was carried out at 37° for 24 h, followed by extraction with 1 *N* sodium hydroxide solution. The organic phase was washed and evaporated; the alkaline phase was subjected to enzymatic hydrolysis as described above.

Derivative formation

Methyloximes (MO) were prepared according to Gardiner and Horning[21]; butyloximes (BuO) and isopentyloximes (*i*PO) as described by Baillie *et al.*[22], and

benzyloximes (BO) following Devaux *et al.*[23]. Benzyloxime trimethylsilyl ethers (BO-TMS) were prepared from the oximes by treatment with bistrimethylsilyltrifluoroacetamide (BSTFA).

Gas–liquid chromatography (GLC)

A Carlo Erba (Milan, Italy) Model GV instrument was used with W-shaped 12-ft. (3 mm I.D.) glass columns, prepared and packed with 1% stationary phase (OV-1 or OV-101), on silanized Gas-Chrom P (Applied Science Labs., State College, Pa., U.S.A.), according to Horning *et al.*[24]. Separations were carried out with temperature programming from 190 to 300° at 1.2°/min; *n*-alkanes (C_{24}–C_{38}) were used to calculate retention data, expressed as methylene units (M.U.) for the steroids studied[24]. Cholesteryl decylate was added as an internal standard to the extracts when analysed as BO-TMS.

Gas chromatography–mass spectrometry (GC–MS)

This was carried out using an LKB-9000 instrument, with an electron energy of 70 eV, a molecular separator temperature of 250° and a source temperature of 270°. Single-ion monitoring was carried out at 28 eV.

Gel chromatography

Sephadex G-25 (Superfine, 10–40 μm; Pharmacia, Uppsala, Sweden) was fractionated by an aqueous sedimentation method[25] to give particles of uniform diameter. Hydroxyalkoxypropyl derivatives were prepared from each fraction by the method of Ellingboe *et al.*[26]. Gravity flow columns (100 or 50 cm × 0.6 cm I.D.) were constructed and packed with a lipophilic gel containing 56% by weight of hydroxyalkyl residues, according to published methods[27,28]. Benzene and methanol–*n*-heptane (9:1, v/v) were used as eluents for straight-phase or reversed-phase systems, respectively. Samples were dissolved in 25–50 μl of chloroform and were applied as layers to the top of the gel bed. In the straight-phase chromatography of urinary extracts, the eluent was changed to benzene–isopropanol (3:1, v/v) after six column volumes of benzene had passed. This solvent mixture resulted in a gel filtration system[29] and all polar material remaining on the column was eluted in less than one bed volume. Columns were subsequently regenerated by passing three column volumes of benzene. Fractions of eluate, volume 5–10% of the column bed, were collected and samples detected by analytical thin-layer chromatography (TLC) or by GLC following derivative formation. Benzyloxime derivatives chromatographed in the reversed-phase system were detected in some cases using the LKB Uvicord III (LKB, Stockholm, Sweden) ultraviolet detector at wavelengths of 206, 254 and 280 nm.

Synthesis of reference steroids

3α,15α-Dihydroxy-5-androstene-17-one (15α-OH-DHA) and 5-androstene-3β, 15α,17β-triol. A biological hydroxylation was carried out using the microorganism *Fusarium graminearum* (Central Bureau voor Schimmelcultures, Baarn, The Netherlands) according to Urech *et al.*[29] with 3β-hydroxy-5-androstene-17-one (DHA) as the substrate. The major product, isolated by TLC, was shown to be an androstenediolone by GLC and GC–MS. The position of hydroxylation was confirmed as 15α by the 250-MHz NMR spectrum and by chromic acid oxidation which yielded a

product with melting point and UV spectrum characteristic of 5-androstene-3,15,17-trione[30]. Sodium borohydride reduction of the diolone in methanol gave 5-androstene-3β,15α,17β-triol.

7-Oxo and 7-hydroxy-Δ5 compounds. From DHA, the method described by Stodola *et al.*[31] was followed to yield 5-androstene-3β,16β,17β-triol. The acetylated product was oxidized with chromic acid, and 3β,16β,17β-trihydroxy-5-androstene-7-one was obtained following saponification. Starting from 5-androstene-3β,17β-diol, an analogous reaction scheme yielded 3β,17β-dihydroxy-5-androstene-7-one; borohydride reduction yielded a mixture of the corresponding 7α- and 7β-triols.

RESULTS AND DISCUSSION

GLC analysis of total neutral urinary steroid extracts yielded retention values and GC–MS data for the quantitatively prominent individual constituents. The major known metabolites were readily identified in these profiles and characterization of the principal unknown structures was possible[3,9,10,23]. However, many GLC peaks constituted unresolved mixtures of several components. GLC and GC–MS study of the metabolites contained in the separated sulpho- and glucurono-conjugated fractions was a first step in simplifying these complex mixtures. Particular interest lies in this approach as the two forms of conjugation were shown to be of different biological significance: the glucuronide fraction was found to contain mainly saturated pregnane metabolites, most probably of placental origin, disappearing after the first week of life, and the sulpho-conjugated metabolites could be considered as representing the characteristic newborn steroid production (mostly Δ5,3β-hydroxylated steroids)[3]. The GLC separation of a sulphate fraction as BO-TMS is shown in Fig. 1. Although the use of BO-TMS afforded obvious advantages with regard to both resolution and structural information[23], GC–MS data demonstrated that composite GLC peaks were

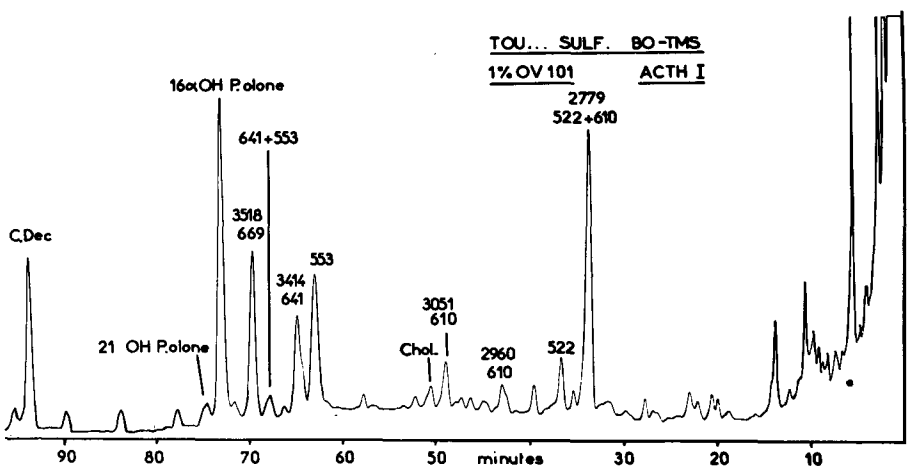

Fig. 1. GLC separation of the BO-TMS derivatives of sulpho-conjugated urinary steroid metabolites contained in the urine of newborn infants, aged 3 days. M.U. and molecular ion values are indicated for the major characterized metabolites (see Table I).

TABLE I

GLC (M.U. VALUES) AND GC–MS (MOLECULAR ION M^+) PARAMETERS OF THE MAJOR STEROID METABOLITES (BO-TMS DERIVATIVES) CHARACTERIZED IN THE SULPHATE FRACTION IN A URINE POOL OF NORMAL NEWBORN INFANTS AGED 1–5 DAYS

M.U. (OV-1, BO-TMS)	*M^+ (GC–MS)*	*Metabolite*
25.56	434	5-Androstene-3β,17α-diol, di-TMS
27.79	522	5-Androstenetriol, tri-TMS
	610	5-Androstenetetrol, tetra-TMS
28.40	522	5-Androstene-3β,16α,17β-triol, tri-TMS
28.98	522	Pregnanetriol, tri-TMS
29.60	610	5-Androstenetetrol, tetra-TMS
30.51	550	Pregnenetriol, tri-TMS
	610	5-Androstenetetrol, tetra-TMS
30.84	550	5-Pregnene-3β,20α,21-triol, tri-TMS
33.74	553	5-Androstene-3β,16α-diol-17-one, BO-di-TMS
34.14	553	5-Androstene-3β,16β-diol-17-one, BO-di-TMS
	641	Androstenetriolone, BO-tri-TMS
34.80	553	5-Androstene-3β,17β-diol-16-one, BO-di-TMS
	641	Androstenetriolone, BO-tri-TMS
35.18	669	Pregnenetriolone, BO-tri-TMS
35.92	581	5-Pregnene-3β,16α-diol-20-one, BO-di-TMS
	641	Androstenetriolone, BO-tri-TMS
36.27	581	5-Pregnene-3β,21-diol-20-one, BO-di-TMS

still present containing components of unknown structure. This is illustrated in Table I, where GC–MS data are listed for the major individual metabolites characterised in the sulphate fraction of a urine pool from 1–5-day-old infants. Similar data for the glucuronide fraction were obtained and have been described previously[3].

To simplify these biological mixtures further, we have investigated lipophilic gel chromatography as a suitable method for the group separation of urinary steroids prior to GLC and GC–MS and for the purification of samples for mass fragmentography.

Straight-phase gel chromatography

A straight-phase gel column was prepared using a hydroxyalkoxypropyl Sephadex derivative[26] with benzene as eluent. Approximate values for the standard elution volume (SEV, *i.e.*, the elution volume relative to the column bed volume multiplied by 100)[27] were measured for a series of model compounds (Table II). Because of the qualitative method used for sample detection in the column eluent, only approximate SEV data were obtained. As we were concerned primarily with the separation of groups of steroids, this accuracy was sufficient. Mixtures of up to ten model compounds could be passed simultaneously and the components distinguised in column fractions by TLC. Examination of the data shows that the elution volume depends largely on the number of hydroxyl groups in the compound and not on their position or orientation. The separation processes occurring in the gel column appear to be those of adsorption, partition and gel filtration[15]. Solute adsorption on the gel is

TABLE II

ELUTION DATA FOR MODEL COMPOUNDS IN THE STRAIGHT-PHASE (BENZENE) SYSTEM AND THE PROPOSED FRACTION BOUNDARIES FOR URINARY STEROID EXTRACTS

The polar fraction (SEV >600) was eluted with benzene–isopropanol (3:1, v/v).

SEV range	*Model compounds used in calibration*	*Approximate SEV values*
0–30	Column void volume	
30–80	*Non-polar fraction*	
	4-Androstene-3,17-dione	60
	4-Pregnene-3,20-dione	60
80–150	*Monohydroxy fraction*	
	3α-Hydroxy-5α-androstane-17-one	90
	3β-Hydroxy-5-androstene-17-one	90
	3α-Hydroxy-5β-androstane-17-one	90
	17β-Hydroxy-4-androstene-3-one	110
	20β-Hydroxy-4-pregnene-3-one	80
150–250	*Dihydroxy fraction*	
	5-Androstene-3β,17β-diol	210
	3β,15α-Dihydroxy-5-androstene-17-one	185
	3β,15α-Dihydroxy-5α-androstane-17-one	190
	3β,16α-Dihydroxy-5-androstene-17-one	175
	3β,17β-Dihydroxy-5-androstene-7-one	220
	5β-Pregnane-3α,20α-diol	150
	3β,16α-Dihydroxy-5-pregnene-20-one	175
	3β,17α-Dihydroxy-5-pregnene-20-one	175
250–600	*Trihydroxy fraction*	
	5-Androstene-3β,16β,17β-triol	450
	5-Androstene-3β,7α,17β-triol	400
	5-Androstene-3β,7β,17β-triol	400
	3β,16β,17β-Trihydroxy-5-androstene-7-one	600
	5β-Pregnane-3β,17α,20α-triol	280
>600	*Polar fraction*	

normally considered to be the result of hydrogen-bond formation between the ether linkages of the gel network and suitable hydrogen donors in the solute[32,33]. Thus, hydroxylic, amino and acidic compounds are retarded relative to hydrocarbons, ketones and esters on straight-phase columns eluted with non-polar solvents such as benzene. Points of subdivision can be delineated on the SEV scale separating groups of steroids on the basis of the number of hydroxyl functions present in the molecules. Elution data for a variety of different compounds in this system have already been published[27,28,33] and most of these compounds are eluted within the appropriate SEV range. Exceptions which have so far been found, for example 7α- and 7β-hydroxycholesterol (SEV 100 and 117, respectively), are unlikely to be present in the urine of newborn infants as major metabolites. The boundaries indicated in Table II for the group separation of steroid metabolites in the urine of newborn infants are proposed on the basis of data so far obtained and it is possible that adjustments may be necessary to deal with extracts containing mixtures of a different composition. Additional

fractions may be included in the overall scheme. Thus phospholipids are eluted close to the column void volume (for example lecithin, SEV 35–40) and triglycerides appear before SEV 50 (for example, tristearin, SEV 42.2). During chromatography of plasma steroids, therefore, an additional fraction, SEV 30–50, might be introduced to remove phospholipid and triglyceride contaminants from the non-polar steroid fraction.

Reversed-phase chromatography

Many of the steroidal metabolites of interest in the urine of newborn infants are polar compounds. A reversed-phase chromatographic system, in which compounds are eluted in inverse order of polarity, would be of value for their isolation and purification. Hydroxyalkoxypropyl-Sephadex has been used previously in systems of this type, with polar organic solvents as eluents[26,34]. Normally, solvents of lower polarity are added to the eluent as modifiers. When very polar samples are chromatographed, it is often necessary to add water to the eluent[26] or to decrease the polarity of the gel phase[16] in order to obtain a favourable partition of the solute between the mobile and stationary phases. Alternatively, the sample polarity can be decreased by derivative formation, for example by esterification, allowing less polar eluents to be used[16].

A mixture of model compounds, of polarity ranging between 4-androstene-3,17-dione and 5β-pregnan-3β,17α,20α-triol, was chromatographed on hydroxyalkoxypropyl-Sephadex gel, eluted with methanol–*n*-heptane (9:1, v/v). Virtually no separation was observed of the components of the mixture, which were all eluted in the range 40–50 SEV units. The mobile phase appeared to be favoured over the non-polar stationary phase in the partition process.

The model compounds were re-chromatographed following the preparation of oxime derivatives, which, in general, resulted in a decrease in polarity of the compounds. For comparison purposes, benzyl-, methyl-, *n*-butyl- and isopentyloximes were chromatographed. Elution data are summarised in Table III. For each type of oxime, three regions can be distinguished on the SEV scale, within which are eluted compounds of high, medium and low polarity. Diols, diolones, triols and triolones are eluted first, followed by monohydroxyketones and then diketones. As the size of the lipophilic oxime substituent is increased, the degree of retardation on the column is more noticeable. However, the decrease in polarity following oxime formation appears to be insufficient to change noticeably the elution volumes of dihydroxy- and trihydroxyketones from those of the non-derivatized steroids.

In preliminary experiments, it was found that excess of benzylhydroxylamine hydrochloride reagent was eluted before the polar steroid fraction, thus providing a convenient purification procedure which, in terms of sample recovery, might be preferable to a solvent extraction procedure. The solvent mixture used in this system permits sample detection with an ultraviolet detector, which is particularly advantageous for the case of benzyloximes. An analogous system has recently been described for use with non-UV-absorbing hydroxylic steroids[35].

Applications to urine of newborn infants

GLC analysis of the diol fraction obtained by straight-phase gel chromatography of a newborn's urine sample is illustrated in Fig. 2. The three major components were identified by GC–MS of the BO-TMS derivatives. The more polar triolone contaminants, which are not resolved from these diolones by GLC of the total

TABLE III

ELUTION DATA FOR STEROID OXIME DERIVATIVES IN THE REVERSED-PHASE SYSTEM

Compound	*Elution volume range (SEV units)*			
	BO	*MO*	*BuO*	*iPO*
5β-Pregnan-3β,17α,20α-triol	40–45	40–45	40–45	45–50
3β,16α,17β-Trihydroxy-5-androstene-7-one	40–50	45–50	—	—
5β-Pregnan-3β,20α-diol	45–50	45–50	45–55	55–60
5-Androstene-3β,17β-diol	45–50	45–50	—	65–70
3β,17α-Dihydroxy-5-pregnene-20-one	55–65	50–58	55–66	80–90
3β,15α-Dihydroxy-5-androstene-17-one	40–45	—	—	—
3β,17β-Dihydroxy-5-androstene-7-one	45–50	45–55	—	—
3β-Hydroxy-5-androstene-17-one	65–75	58–66	66–77	90–100
3α-Hydroxy-5β-androstane-17-one	65–70	58–66	66–77	90–100
17β-Hydroxy-4-androstene-3-one	70–80	58–66	66–77	90–100
21α-Hydroxy-4-pregnene-3-one	80–90	66–82	71–88	100–110
4-Androstene-3,17-dione	120–130	86–106	120–130	155–175
4-Pregnene-3,20-dione	185–195	106–140	—	255–285

sulphate fraction (see Table I), were absent. This approach is currently used to obtain a group separation of urinary steroid metabolites in newborn infants prior to GC–MS study. Sephadex LH-20 has been used for a similar purpose[8,17], however highly substituted hydroxyalkoxypropyl-Sephadex appeared more flexible in its use with various solvent systems and the behaviour of steroid metabolites can be predicted on a structural basis. An alternative approach is provided by reversed-phase systems which can be used to achieve a rapid purification of the quantitatively important polar di- and trihydroxysteroids.

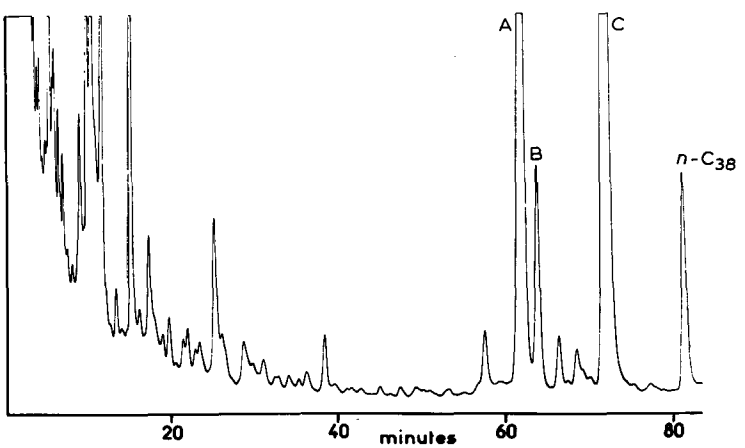

Fig. 2. GLC separation of BO-TMS derivatives of dihydroxysteroids isolated from an extract of sulpho-conjugated urinary steroids by chromatography on a straight-phase gel column (50 cm × 0.6 cm I.D.) eluted with benzene. A = 16α-OH-DHA: M.U., 33.74; M^+, 553. B = 16β-OH-DHA: M.U., 34.14; M^+, 553. C = 16α-OH-pregnenolone: M.U., 35.92; M^+, 581.

The diol fraction (as BO-TMS) illustrated in Fig. 2 was examined for the presence of 15α-OH-DHA by mass fragmentography at *m/e* 553, 463 and 356 and it was concluded that this compound was probably present in the urine of newborn infants at a level of 5–10 μg per 24 h (ref. 3). A total sulphate fraction was examined by single-ion monitoring at *m/e* 463 (base peak in the mass spectrum of 15α-OH-DHA). The trace obtained can be compared with that given by the diol fraction (Fig. 3). Obviously, gel fractionation was very effective in removing many interfering peaks which obscured the detection of the metabolite in the crude extract.

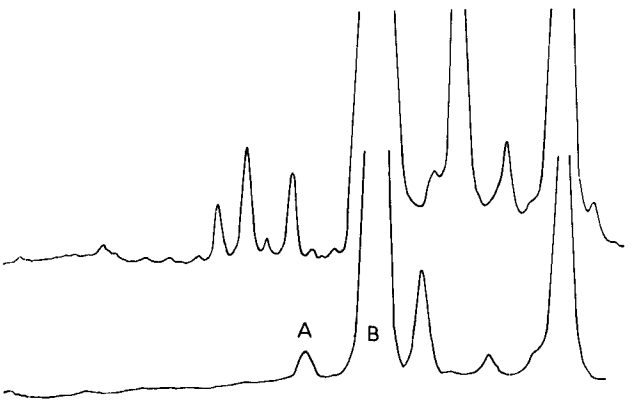

Fig. 3. Drawing of single-ion monitoring traces at *m/e* 463 for a total steroid sulphate fraction (upper) and for the diol fraction illustrated in Fig. 2 obtained by lipophilic gel chromatography. Peaks A and B had the retention time of 15α-OH-DHA and 16α-OH-DHA, respectively. Both samples were analysed as BO-TMS on a 3-m 1% OV-1 column with temperature programming at 1.5°/min from 200 to 290°.

CONCLUSIONS

Highly substituted lipophilic gels were evaluated for the purification and fractionation of urinary steroid extracts and straight-phase and reversed-phase solvent systems were developed. Both systems are suitable as a preliminary separation method for GLC or GC–MS analysis, and they provide a potential source of additional information on the structure of the urinary metabolites. The first chromatographic system was found to be very convenient for group separation of steroid metabolites. In the reversed phase, oxime formation improves the separation of the polar compounds and, in the case of benzyloximes, facilitates sample detection using a UV detector.

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