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NEW BIOCHEMICAL SEPARATIONS USING PRECOLUMN DERIVATIZATION AND MICROCOLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

Microcolumn liquid chromatography with slurry-packed capillary columns has been used to resolve mixtures of biologically important steroids and prostaglandins. In order to enhance detection sensitivity, the samples are first derivatized with a suitable chromophore-yielding agent. Hydroxy steroids were derivatized with either benzoyl chloride or 7-(chlorocarbonylmethoxy)-4-methylcoumarin, while Dns hydrazine was employed to react with the ketonic groups of certain steroidal conjugates. Bile acids can also be derivatized with bromomethylcoumarin to yield fluorescent products. The use of microcolumns permits both a high degree of component resolution and enhanced detection sensitivity.

INTRODUCTION

The ever-increasing importance of biological and biochemical investigations demands analytical separation techniques with great resolving power and highly sensitive detection. Over the last 20 years, gas chromatography (GC) has found an increasing number of applications in biochemistry and medicine'. The high separation efficiency of GC and the availability of both universal and selective detection means have enabled development of the multicomponent analysis concept[2,3] for relatively volatile biological substances; numerous applications now attest to the general validity of this concept of "metabolic profiling".

Many biologically important molecules are either too large or too polar to be effectively analyzed by GC. Although some of these problems have been overcome through a variety of derivatization methods[1,4], many investigators are still somewhat uncomfortable about using gas-phase analytical techniques for quantitative purposes. Liquid chromatography (LC) is considered to be "a more natural method" for such determinations. However, contemporary LC, with typical numbers of theoretical

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plates between 10^3 and 10^4, frequently fails to achieve the necessary component resolution. Therefore, use of high-performance liquid chromatography (HPLC) in resolving complex mixtures is uncommon.

Initially, one of the incentives for developing microcolumn LC was its potential for high-efficiency separations[5–8]. Although much remains to be done in approaching the ultimate potential of microcolumns, recent results on slurry-packed capillary columns[9–11] fully demonstrate their capability in resolving complex mixtures. The numbers of theoretical plates obtained at adequate capacity ratios now approach those commonly observed in modern capillary GC.

Unfortunately, the detection problems of modern LC still place this otherwise powerful analytical method at a disadvantage. Only certain biological molecules possess structural features which result in UV absorption, natural fluorescence, or electrochemical activity. For example, only some mammalian acidic metabolites are aromatic, while others remain undetected by either a UV or a fluorescence detector. Likewise, in the series of metabolically important steroids, only the phenolic steroids (estrogens) and those with double-bond conjugation in the A-ring are readily detectable. However, as different molecules within a class of biologically interesting substances often share a common functionality, relatively simple derivatization methods may be developed to uniformly "tag" these molecules so that conventional detectors can be used.

Postcolumn derivatization methods are generally confined to very fast reactions[12], limiting their scope of application for a variety of biological molecules and derivatization agents. In addition, their use in microcolumn LC is severely limited by the need for minimal volumes between the column end and the point of detection. Consequently, precolumn derivatization appears more meaningful for use in micro-LC. The often-raised objection to this approach is the loss of functional groups that might participate in selectivity-based separations. However, this problem can be overcome by greater column efficiencies generated through the microcolumn approach.

The purpose of this communication is to describe certain applications of the novel microcolumn techniques to biochemical investigations. Although some results are still preliminary, it appears that general derivatization schemes can be developed to "tag" important classes of biological substances for highly efficient multicomponent determinations using microcolumn LC. The examples shown in this report include the benzoylation of steroids[13] for UV detection, and the reaction of certain steroidal conjugates with Dns hydrazine, as well as the derivatization of bile acids with bromomethylcoumarin for spectrofluorimetric detection. In addition, a generally applicable reagent, 7-(chlorocarbonylmethoxy)-4-methylcoumarin, was developed[14] to derivatize hydroxy compounds for high-sensitivity fluorescence detection.

EXPERIMENTAL AND RESULTS

Instrumentation

The principal components of miniaturized liquid chromatographs which were modified for use with capillary columns were previously described[5,10,11]. The detectors used were either the Jasco Uvidec-lOO-IV ultraviolet monitor or the Schoeffel FS 970 fluorometer. Detection cells were modified with a fused-silica capillary, to have approximate detection volumes of 10 nl and 160 nl, respectively. The sample

introduction technique and features of the stepwise gradient have also been described[11]. In some separations, a miniaturized continuous-gradient device[15] was employed.

Columns of different lengths were prepared by packing flexible fused-silica capillaries with a slurry of 3 or 5 μm spherical particles (Phase Sep, Norwalk, CT, U.S.A.), as described in a previous communication[10]. The flow-rates used for the separations described were *ca.* 1 μl/min.

Benzoylation of steroids

As shown previously by Fitzpatrick and Siggia[15], hydroxy steroids react easily with benzoyl chloride to form UV-absorbing derivatives. Approximately 50 μl of benzoyl chloride usually suffices[13] for the complete derivatization of steroids at concentrations below 10^{-6} g. Alternatively, a larger volume of reagent may be used, followed by the removal of excess reagent and by-products on a C_{18} Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.), with 45% acetonitrile in water. The derivatized steroids are subsequently eluted with a volume of methanol, evaporated

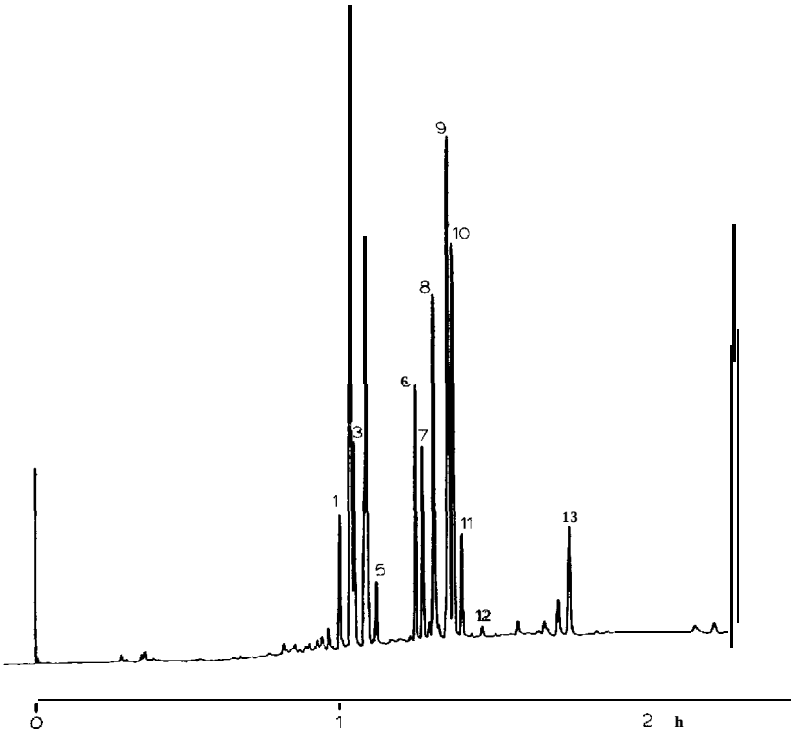

Fig. 1. Reversed-phase chromatography of benzoylated hydroxy steroids under the conditions of stepwise gradient: 80% acetonitrile (ACN) in water (15 minj; 85% ACN in water (14 min); 90% ACN in water (15 min); 95% ACN in water (18 min); 100% ACN. Column, 1 m × 240 μm I.D. fused-silica capillary packed with 3-μm ODS particles. Peaks: 1 = 11-hydroxyandrosterone; 2 = 11-hydroxyetiocholanolone; 3 = *allo*-tetrahydrocortisol; 4 = tetrahydrocortisol; 5 = tetrahydrocortisone; 6 = β-cortolone; 7 = β-cortol; 8 = a-cortolone; 9 = α-cortol; 10 = etiocholanolone; 11 = androsterone; 12 = dehydroepiandrosterone; 13 = pregnanetriol; 14 = androstanediol.

to dryness, redissolved in the desired solvent, and applied to a slurry-packed microcolumn. The wavelength used for detection was 230 nm.

An efficient separation of the common standard steroids is shown in Fig. 1; closely related steroids, such as 11-hydroxyandrosterone and 11-hydroxyetiocholanolone (peaks 1 and 2), *allo*-tetrahydrocortisol and tetrahydrocortisol (peaks 3 and 4), and etiocholanolone and androsterone (peaks 10 and 11), are resolved from each other. In addition, β-cortolone and β-cortol, which normally co-elute in capillary GC, are separated (peaks 6 and 7).

Given the usual structural features of biologically important steroids (17-ketosteroids, progesterone metabolites, corticosteroids, etc.), the degree of compound retention in a reversed-phase system will primarily be determined by the reactivity

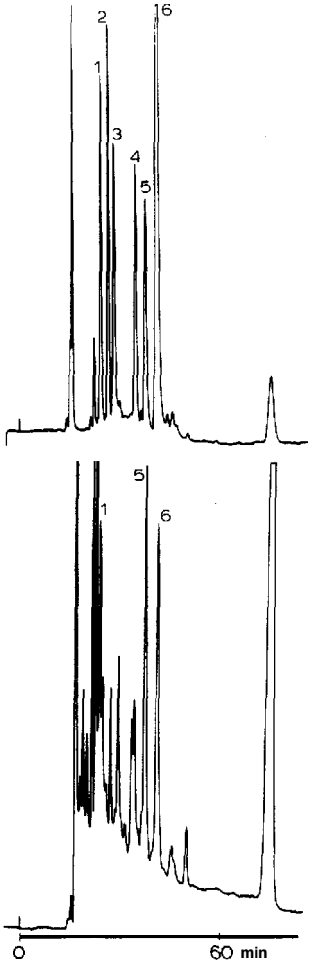

Fig. 2. Separation of steroid conjugates as Dns derivatives; isocratic run, 0.01 *M* sodium acetate in methanol-water-acetic acid (65:35:1); column, as given in text. Peaks: 1 = 11-hydroxyandrosterone; 2 = androsterone sulfate; 3 = Δ^5-androstenone glucuronide; 4 = testosterone glucuronide; 5 = etiocholanolone glucuronide; 6 = androsterone glucuronide. Top chromatogram corresponds to standards, the bottom chromatogram to an aliquot of normal male urine.

of different hydroxy groups and by the hydrophobicity of the resulting derivative. Preparation of benzoyl derivatives was found to be quantitative and reproducible, permitting an application of this technique to profiling the urinary steroid metabolites in human diabetic conditions[13].

Reaction of steroid conjugates with Dns hydrazine

The recently published method of Kawasaki *et al.*[17] was developed to measure intact steroid conjugates (mainly glucuronides and sulfates) in biological fluids. A fluorescence-yielding reagent, dansyl hydrazine, is allowed to react with 17-oxosteroids, while the conjugate moieties of these molecules remain unhydrolyzed.

We have adapted this procedure[17] to the conditions of microcolumn chromatography. In accordance with the original procedure, a 10-ml aliquot of human urine is first passed through a Sep-Pak C_{18} cartridge, which is further washed with water and 20% aqueous methanol. The retained steroids are subsequently eluted with methanol, evaporated to dryness and derivatized with a Dns hydrazine solution as previously described[17]. The sample is then introduced in a methanolic solution onto a 1 m × 250 μm I.D. microcolumn, packed with 5-μm Spherisorb C_{18}. Prior to

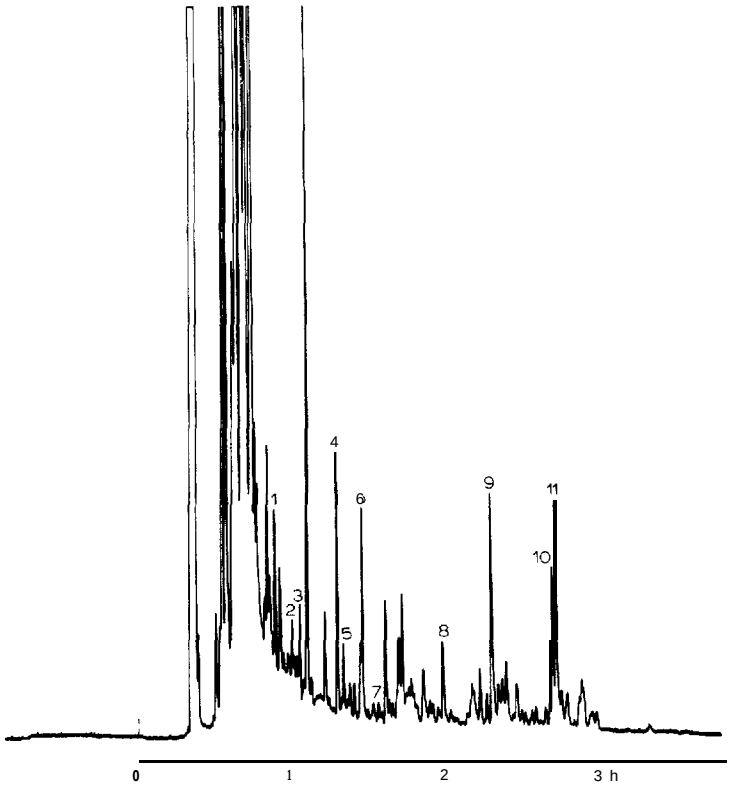

Fig. 3. Chromatogram of the derivatized sample of solvolyzed plasma steroids. Continuous gradient, 75100% acetonitrile-water. Column, as indicated in text. Tentatively identified components: 1 = 11β-hydroxyetiocholanolone; 2 = tetrahydrocortisol; 3 = β-cortolone; 4 = androsterone; 5 = dehydroepiandrosterone; 7 = pregnanetriol; 8 = pregnanediol; peaks 6 and 9–11 appear to be polyderivatizcd androstane derivatives.

sampling, the excess derivatization agent is removed with 2-heptanone. Separation of standard conjugates and the urinary extract components is seen in Fig. 2, where the last peak corresponds to a derivative of 2-heptanone.

***Derivatization of hydroxy compounds with** 7-(chlorocarbonylmethoxy)-4-methylcoumarin*

A new fluorescence reagent, 7-(chlorocarbonylmethoxy)-4-methylcoumarin, has been synthesized and examined in this laboratory[14] in order to derivatize a variety of hydroxy compounds. To permit high-sensitivity detection, a major goal of this research has been to prepare first derivatives whose excitation maxima are near the 325 nm output of a heliumcadmium laser fluorescence detector[18]. The new reagent provides this property.

While the reactivity of this reagent and the quantitative aspects of its use are described elsewhere[14], two applications of this derivatization principle are illustrated here: the detection of solvolyzable plasma steroids and the separation of standard prostaglandins.

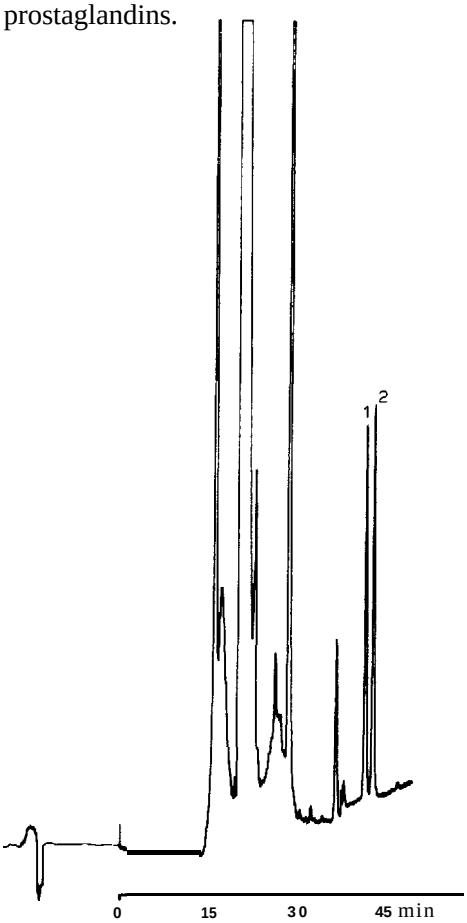

Fig. 4. Separation of two derivatized prostaglandins. Continuous gradient, 65–100% acetonitrile in water (1% acetic acid), Column, 1 m × 240 μm I.D. fused-silica capillary packed with 5-μm ODS particles. Peaks: 1 = PGA_1; 2 = PGE_1.

A human serum sample was analyzed for solvolyzable steroids using a procedure described by Axelson and Sahlberg[19], which consists of the initial isolation of steroid sulfates on a Sep-Pak cartridge and the subsequent solvolysis of the methanol-eluted steroid conjugates. The sample is then further purified with ion-exchange Sephadex materials and derivatized with the fluorescence reagent[14]. A chromatogram of these plasma steroids, obtained with a 1.5 m × 240 μm I.D. fused-silica column packed with 3-μm ODS particles, is shown in Fig. 3; several of the major profile constituents have been tentatively identified through mass spectrometry.

Besides its obvious potential utility in steroid analysis, 7-(chlorocarbonylmethoxy)-4-methylcoumarin can also be used to provide quantitative fluorescent derivatives for a variety of other hydroxy compounds which occur so widely in natural samples. In this direction, a preliminary result is shown in Fig. 4, which demonstrates the applicability of this procedure to the separation and detection of prostaglandins.

Derivatization of bile acids with bromomethylcoumarin

The modified procedure of Okuyama *et al.*[20] was used to derivatize standard bile acids. The bile acids (0.1 μg each) were dissolved first in *60* μl of acetonitrile,

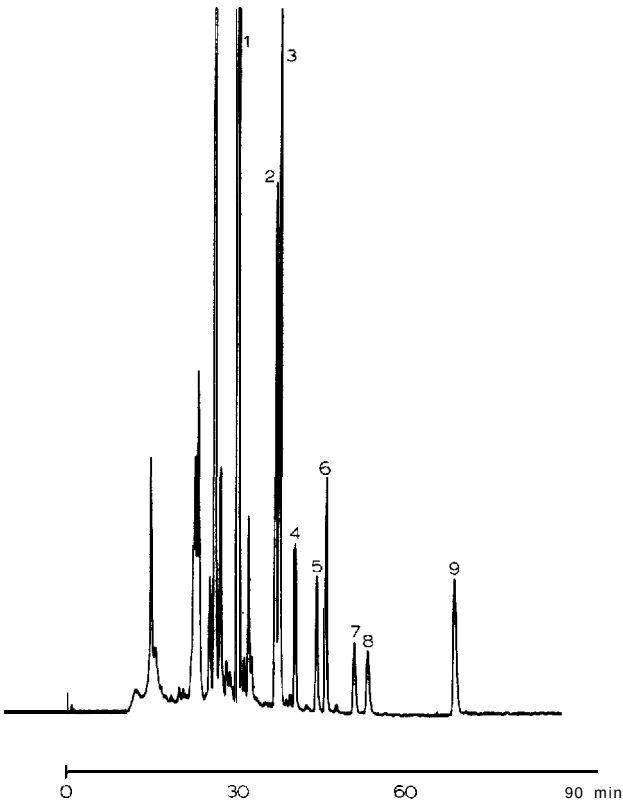

Fig. 5. Chromatogram of bile acid standards. Column, 1 m × 240 pm I.D. fused-silica capillary packed with 5-μm Spherisorb ODS; stepwise gradient, 70–100% acetonitrile in water. Peaks: 1 = glycocholic acid; 2 = glycochenodeoxycholic acid; 3 = glycodeoxycholic acid; 4 = cholic acid; 5 = uro-deoxycholic acid; 6 = glycolithocholic acid; 7 = deoxycholic acid; 8 = chenodeoxycholic acid; 9 = lithocholic acid.

while 20 μl of a crown ether catalyst (20 mg/ml in acetonitrile), a few grains of potassium carbonate, and 20 μl of bromomethylcoumarin (20 mg/ml in acetonitrile) were added. The reaction mixture was then heated to 80°C for 15 min. It was further partitioned between chloroform and water to remove the reaction by-products, and the chloroform layer was analyzed. A chromatogram of nine bile acid standards is shown in Fig. 5.

DISCUSSION

Improved separations of biological compounds, such as nucleotides and bases[21], or amino acids and peptides[22], were among the first accomplishments of HPLC during the 1960s. However, earlier attempts to use modern ion-exchange LC for recording complex metabolic patterns in human diseases[23–25] were only partially successful because of the relatively long analysis times and the lack of suitable detection techniques. Some improvements were later realized through an application of reversed-phase LC methods to ionic substances[26,27].

Significant improvements in column performance were recently achieved through microcolumn separation techniques. Using slurry-packed capillary columns'*, efficiencies above 200,000 theoretical plates can now be achieved in analysis times of less than 10 h[28]. The present publication shows several practical examples of the use of microcolumn techniques in biochemical separations. Although a need for high plate numbers may vary with the application, the general capabilities for multicomponent profiling work have now been enhanced.

Improved detection capabilities are yet another important reason for choosing microcolumns in certain biochemical applications. While using concentration-sensitive detectors, a simultaneous reduction in the column diameter (and, correspondingly, flow-rate) with the detection volume results in significantly enhanced mass sensitivity[13,29]. Both UV and fluorescence spectroscopic detectors benefit from this component miniaturization. In addition, impressive sensitivities were recently shown with miniaturized electrochemical detectors[30–32].

Since universal and sensitive detectors are still unavailable in the practice of modern LC, sample derivatization techniques and selective detection are essential to many future applications. We have shown in this publication that precolumn derivatization and microcolumn LC can effectively be combined to expand certain analytical uses of these techniques. Although the popularity of sample derivatization prior to LC has been steadily on the rise, the general scope is likely to expand further with more emphasis on multicomponent biological determinations. While the examples chosen for this publication concern only steroids and prostaglandins, other biological determinations may also be developed in a similar manner.

These recent advances in component resolution and sensitive detection through microcolumn LC techniques provide further incentive toward the development of on-line methods for solute identification. Ancillary techniques for microcolumn LC, which would be equivalent in identification power to capillary GC combined with mass spectrometry or Fourier-transform infrared spectroscopy, are thus far underdeveloped.

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