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Journal of Chromatography A, 869 (2000) 243–250

JOURNAL OF CHROMATOGRAPHY A

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2-(4-Carboxyphenyl)-6-*N*,*N*-diethylaminobenzofuran: a useful reagent for the sensitive determination of alcohols by high-performance liquid chromatography with fluorimetric detection

Peter Assaf[a], Jehoshua Katzhendler[b], Abdullah I. Haj-Yehia[a,*]

[a]*Department of Pharmaceutics, The David R. Bloom Center for Pharmacy, School of Pharmacy, Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem 91120, Israel*

[b]*Department of Medicinal Chemistry, The David R. Bloom Center for Pharmacy, School of Pharmacy, Hebrew University of Jerusalem, P.O. Box 12065, Jerusalem 91120, Israel*

Abstract

A simple and highly sensitive method for the determination of short, medium and long-chain alcohols using high-performance liquid chromatography with fluorimetric detection is described. The alcohols were derivatized to their corresponding esters with (4-carboxyphenyl)-6-N,N-diethylaminobenzofuran. The esterification reaction proceeded rapidly and smoothly in acetonitrile at 60°C with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (a coupling agent) in the presence of 4-dimethylaminopyridine (a base catalyst). The resulting esters of alcohols from methanol to eicosanol (C_1–C_{20}-ol) were separated on a reversed-phase column (Ultrasphere C_8) with gradient elution (acetonitrile–water) and detected fluorometrically (excitation 387, emission 537 nm). The lower limits of detection (signal-to-noise ratio of 3) for the derivatized alcohols were in the range of 0.2–0.5 pg. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Derivatization, LC; Alcohols; Carboxyphenyldiethylaminobenzofuran

1. Introduction

Despite recent developments in the field, the determination of many drugs and naturally occurring compounds through their hydroxyl group remains a challenging subject. In fact, sensitive quantification of fatty alcohols, sterols and hydroxysteroids in bacteria, fungi and humans has always been a difficult analytical problem. Such quantification may provide valuable information concerning the lipid content, as well as concerning the functional properties of membranes [1–4]. In addition, alcohols with chain lengths from C_8 to C_{20} are widely used in the pharmaceutical and cosmetic industries, and as raw materials in the manufacture of surfactants [5].

Besides their determination by gas chromatography [6,7], several precolumn derivatization methods have been reported for the determination of alcohols using high-performance liquid chromatography (HPLC). These methods involve derivatization with reagents such as phenylisocyanate [8] and 3,5-dinitrobenzyl chloride [9] for the determination of aliphatic alcohols with ultraviolet (UV) detection.

Sensitive determinations of aliphatic alcohols with fluorimetric detection after precolumn derivatization have also been reported. These include derivatization with 4-dimethylamino-1-naphthoyl nitrile [10], 2-methyl-1,1′-binaphthalene-2′-carbonyl nitrile [11],

*Corresponding author. Tel.: +972-2-675-8607; fax: +972-2-675-7015.

E-mail address: abdullah@cc.huji.ac.il (A.I. Haj-Yehia)

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PII: S0021-9673(99)00948-6

the azide [12] and chloride [13] derivatives of 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxaquinoxaline-2-carboxylic acid, 7-methoxycoumarin-3- and -4-carbonyl azides [14], 4-diazomethyl-7-methoxycoumarin [15], 1-anthroyl- and 9-anthroylnitriles [16]. For most of these derivatizing reagents in general, and the azides and acid chlorides in particular, the derivatization procedure involves heating in anhydrous solvent at 100°C for 40 min and then at 130°C for 60 min [3,4,12,13]. Moreover, most of the above mentioned reagents, both in their native form and as their alcoholic derivatives, are unstable and need special cool and dry storage conditions. Derivatization of alcohols in aqueous conditions with the fluorescent labeling reagent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has also been reported [17]. In addition to its high sensitivity, this method has various advantages over other methods in that the reaction time is very fast and enables the separation of isomeric alcohols. However, the reagent has a narrow excitation and emission difference (75 nm) and, because of its high reactivity, undergoes rapid decomposition in aqueous media. In addition, the separation process is long (1 h per run) and takes place on a heated column following complex gradient elution. In fact, the major disadvantage of this highly sensitive method is the limited reactivity of the reagent towards secondary alcohols and its unreactivity towards tertiary alcohols [17].

We recently reported the determination of alcohols after precolumn derivatization with 2-(4-carboxyphenyl)-6-methoxybenzofuran (CPMB) [18]. The native reagent as well as its primary alcoholic derivatives were very stable in aqueous solutions and the separation conditions were very simple and facilitated the complete resolution of 20 alcohols in the same run within a relatively short time (less than 30 min). However, like AQC, CPMB has a narrow excitation and emission difference (75 nm) and its reactivity towards secondary and tertiary alcohols was not satisfactory.

Here, we report the chemical synthesis and analytical application of 2-(4-carboxyphenyl)-6-*N*,*N*-diethylaminobenzofuran (CPDB) for derivatization of primary, secondary and tertiary alcohols. Because of its dialkylamino aromatic substitution, CPDB has a wide excitation and emission difference (150 nm) and the reaction yields with both secondary and tertiary alcohols were higher than with CPMB. The dependence of the derivatization procedure on reaction conditions (time and temperature), dehydrating (coupling) agent and on the catalyst utilized, as well as the separation conditions for the derivatized alcohols are also described.

2. Experimental

2.1. Chemicals

All chemicals used for the synthesis of the reagent and subsequently for the derivatization procedure were purchased from Aldrich (Milwaukee, USA) and except for drying in a desiccator under vacuum (to remove traces of alcohols) they were used without further purification. Acetonitrile, *N*,*N*-dimethylformamide (DMF), triethylamine and water were all of HPLC grade and were filtered through a 4-μm filter (Rainin, USA) and degassed under vacuum prior to use.

Standard solutions (100 ng/ml) of the alcohols from methyl to eicosyl (C_1–C_{20}-ol) were prepared by dilution with acetonitrile of stock solutions of each alcohol (1 mg/ml) in acetonitrile. For long-chain alcohols (i.e., $C>12$), the stock solutions were prepared by dissolving the alcohol in hot DMF and diluting with acetonitrile to a concentration of 10 ng/ml.

The reagent solution (0.01%) was prepared by dissolving 1 mg of CPDB in 0.2 ml of pyridine and diluting to 10 ml with acetonitrile. Solutions of 2 and 4% of the coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and the base catalyst 4-dimethylaminopyridine (DMAP), respectively, were prepared in acetonitrile. The reagent, coupling agent and catalyst-containing solutions were stable for at least 1 week in daylight at room temperature.

2.2. Synthesis of CPDB

To a solution of 4.83 g (25 mmol) of 4-(diethylamino)salicylaldehyde and 4.9 g (25 mmol) of α-bromo-*p*-tolunitrile (4-cyanobenzyl bromide) in DMF (25 ml), there were added dropwise 20 ml of

sodium ethoxide solution [freshly prepared by dissolving 0.6 g (25 mmol) of metal sodium in dry ethanol]. The resulting solution was heated at 120°C for 4 h following which the ethanol was distilled off. The condensed solution was poured into a mixture of ice-cold water (40 g) and methanol (10 ml) and stirred at 0°C for 1 h. The deposited crystals were collected, washed with water and dried in vacuum. The crude product was recrystallized (twice) from methanol to give pure 2-(4-cyanophenyl)-*N*,*N*-diethylaminobenzofuran (CDB), 6.3 g (84%); $UV_{ethanol}$ $\lambda_{max}=400$ nm, (log $\epsilon=4.054$), ^{1}H-NMR ($CDCl_3$): 1.22 (t, 3H, $-CH_2-CH_3$), 3.48 (q, 2H, $-CH_2-CH_3$), 7.12 (s, 1H, Ar–CH=C–Ar), 6.71–7.85 (m, 7H, Ar–H), MS (*m*/*z*) 290 (M^+).

A solution of CDB (1.6 g, 5.5 mmol) and powdered potassium hydroxide (5 g, 90 mmol) in ethylene glycol (50 ml) was refluxed for 8 h, cooled to room temperature and poured into a mixture of ice–water (80 g) and concentrated hydrochloric acid (15 ml). The deposited crystals were collected, washed with water and dried in vacuum. The crude product was dissolved in a minimum of DMF and recrystallized from ethanol to give 1.20 g (70%) of the reagent CPDB; $UV_{ethanol}$ $\lambda_{max}=389$ nm, (log $\epsilon=3.655$), ^{1}H-NMR dimethlysulfoxide (DMSO-d_6): 1.22 (t, 3H, $-CH_2-CH_3$), 3.44 (q, 2H, $-CH_2-CH_3$), 7.12 (s, 1H, Ar–CH=C–Ar), 6.71–8.13 (m, 7H, Ar–H), 10.66 (s, broad 1H, –COOH), MS (*m*/*z*) 308 (M^+).

2.3. Isolation of dodecyl-CPDB

To 50 ml of the stock solution of 1-dodecanol (1 mg/ml) in a round-bottomed flask, 200 mg each of DMAP and EDC were added. The mixture was heated in an oil bath for 30 min at 60°C following which the acetonitrile was evaporated to dryness under reduced pressure. The residue was dissolved in 5 ml of 50% methanol in water and applied to a BondElut C_{18} sample preparation cartridge (Varian, USA). The cartridge was washed three times, each with 3 ml of 50% methanol in water to remove excess reagents. The dodecyl-CPDB ester was eluted with 20 ml of acetonitrile which, upon evaporation to dryness, yielded a white residue. TLC and HPLC analysis showed a single peak (purity >98%). This derivative was used as a standard for studying the effects of the coupling agent, the base catalyst, temperature and solvent on the yield and duration of the derivatization reaction.

2.4. Apparatus

The HPLC system consisted of a Spectra Series P200 pump and a Shimadzu RF 531 fluorescence detector (Shimadzu, Japan) operating at excitation and emission wavelengths of 387 and 537 nm, respectively. Samples were injected either via an autosampler (Spectra Series) or manually using a Rheodyne 20-μl sample loop. Ultraviolet spectra were recorded with a diode array spectrophotometer 8425 A (Hewlett-Packard, USA), and ^{1}H-NMR spectra were recorded on Varian-300 NMR.

2.5. Chromatographic conditions

The HPLC apparatus was connected to an Ultrasphere C_8 column (150×4.6 mm I.D., 5 μm, Beckman, USA). For separation of a mixture of C_1–C_{10}-ol derivatives, the samples were eluted at ambient temperature with water containing 0.1% triethylamine (A) and 0.1% triethylamine in pure acetonitrile (B). A gradient elution from 30 to 100% B in 20 min was used at a flow-rate of 1.5 ml/min for separation of C_1–C_{10}-ol (Fig. 3). A gradient elution from 12 to 100% B in 25 min was used at a flow-rate of 1.5 ml/min for separation of the CPDB derivatives of C_{11}–C_{20}-ol (Fig. 4).

2.6. Derivatization procedure

The derivatization reaction proceeded as shown in Fig. 1: to 2 ml of a standard solution of the alcohols (0.1 ml of each alcohol from C_1 to C_{10} or C_{11} to C_{20}-ol, 5 ng/ml final concentration), were added 2 ml each of DMAP, EDC and the reagent CPDB into a screw-capped tube. The mixture was heated at 60°C for 30 min and then left to cool at room temperature. A 10-μl volume of the crude reaction mixture was injected directly onto the chromatograph.

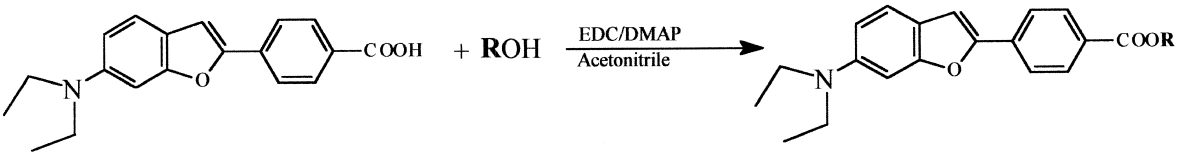

Fig. 1. Scheme for the derivatization reaction of alcohols with CPDB.

3. Results and discussion

3.1. Derivatization conditions

1-Dodecanol was used to study the effects of the reaction solvent, the reagent concentration, the coupling (condensing) agent, the time and the temperature as well as the base catalyst utilized on the fluorescent yield of the product, dodecyl-CPDB ester.

3.2. Solvent effect

Acetone, acetonitrile, chloroform and dichloromethane (and other solvents) were tested for their suitability as reaction solvents for the derivatization procedure. Table 1 shows that dichloromethane and acetonitrile gave the best results as reflected by the detector response. However, acetonitrile is the solvent recommended by us because of the sparing solubility of EDC in dichloromethane and its immiscibility with the mobile phase.

3.3. Base catalyst

Practically no reaction took place without a base catalyst. Table 2 shows that amongst various bases tested, 4-dimethylaminopyridine (4%) gave the highest detector responses. Several concentrations of DMAP were tested. Concentrations in excess of 4% did not increase the reaction yield and were accompanied by unidentified interfering peaks and therefore were avoided. Concentrations of less than 3% of DMAP led to submaximal responses.

Table 1
Effect of solvent on yield (detector response) of the derivatization reaction[a]

Solvent	Detector response (%)
Acetone	27
Acetonitrile	100
Chloroform	80
Dichloromethane	90

[a] Reactions were carried out at 60°C for 30 min with 100 ng/ml of dodecyl alcohol, EDC and DMAP in final concentrations of 2 and 4%, respectively. Each value is an average of six runs with the detector response obtained with acetonitrile taken as 100%.

3.4. Carbodiimide and other coupling agents

Table 3 shows the detector response obtained when various coupling agents were tested in the derivatization reaction (not all listed). Amongst these, EDC hydrochloride gave the best detector response and therefore was selected as the condensing agent for the derivatization procedure. EDC is freely soluble in acetonitrile and concentrations in excess of 2% did not offer any advantage.

3.5. Time and temperature effects

Heat has a significant effect on the reaction time and yield (Fig. 2). When tested at different temperatures over various periods of time, the reaction was

Table 2
Effect of catalyst on yield (detector response) of the derivatization reaction[a]

Base catalyst	Detector response (%)
4-Dimethylaminopyridine (4%)	100
Triethylamine (15%)	20
Pyridine (10%)	17
4-Methylmorpholine (10%)	17
No catalyst	0

[a] Reactions were carried out at 60°C for 30 min with 100 ng/ml of dodecyl alcohol using EDC as the coupling agent (2%) in the presence of the specified final concentration of the base catalyst. Each value is an average of six runs with the detector response obtained with 4-dimethylaminopyridine taken as 100%.

Table 3
Effect of the coupling agent on yield (detector response) of the derivatization reaction[a]

Coupling agent	Detector response (%)
Dicyclohexylcarbodiimide (DCC)	30
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)	100
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide methiodide	45
1-Hydroxybenzotriazole	15

[a] Reactions were carried out at 60°C for 30 min with 100 ng/ml of dodecyl alcohol using 4-dimethylaminopyridine (4%) as the base catalyst in the presence of 2% final concentration of the coupling agent. Each value is an average of six runs with the detector response obtained with EDC taken as 100.

completed within 20 and ≈30 min at 90 and 60°C, respectively. Unidentified by-products were minimal when ester formation was carried out at 60°C. Consequently, this temperature was used throughout. A clean reaction occurred at room temperature, but times in excess of 24 h were needed for a maximal response. Increasing the reagent concentration to more than 0.01% (already a great excess of reagent to alcohol) did not alter the time needed for reaction completion significantly.

3.6. Separation and determination of CPDB derivatives

Fluorescence measurements yielded excitation and emission maxima for dodecyl-CPDB at 387 and 537

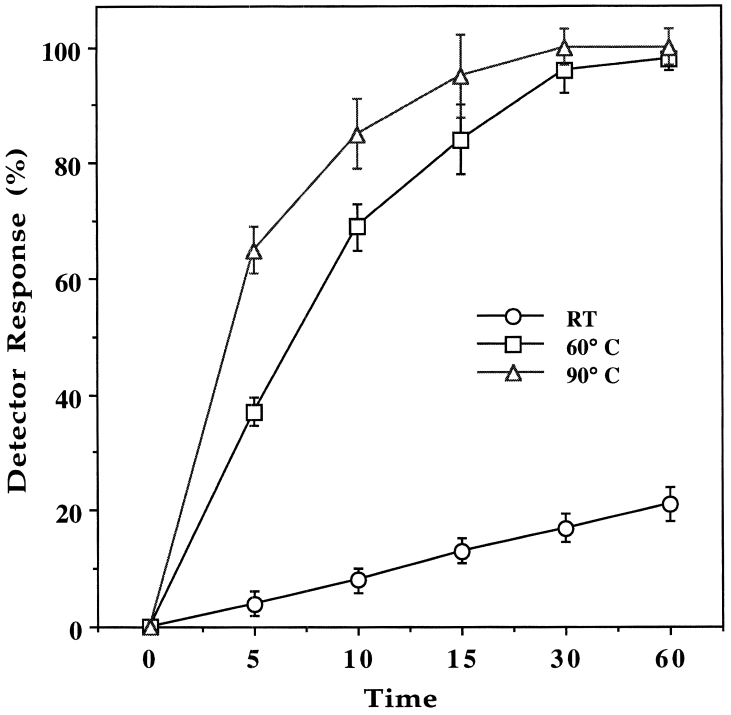

Fig. 2. Effect of temperature on time needed for completion of the derivatization reaction. Reactions were carried out at the specified temperature with 100 ng/ml of dodecyl alcohol in the presence of CPDB, EDC and DMAP at final concentrations of 0.01, 2 and 4%, respectively. Aliquots of the reaction mixture were taken at different time points and injected directly into the chromatograph. Each point represents the mean±S.E of six experiments. Time scale in min.

nm, respectively. When other derivatives were tested (i.e., C_8–C_{16}-ol), similar maximal wavelengths were obtained. The detector was therefore operated at these excitation and emission wavelengths. For the separation of the derivatized alcohols, several pure organic and aqueous organic mixtures were tested. These included methanol, ethanol, isopropanol, DMF, tetrahydrofuran, and acetonitrile in pure form and in aqueous mixture combinations. Several direct and reverse phase columns were tested (silica, C_4, C_8, C_{18}, C_{18}-cyano and C_{18}-amino). For simultaneous separation of alcohols with 1–20 carbon atoms, a C_8 column eluted with a gradient of acetonitrile in water gave the best separation with the shortest retention times. Under these conditions, for example, derivatives of C_1–C_{10}-ol were separated within 20 min (Fig. 3). Similar results were obtained with derivatives of C_{11}–C_{20}-ol (Fig. 4). Triethylamine, (0.1%) was found to be the best organic modifier and gave the sharpest peaks.

3.7. Secondary and tertiary alcohols

The applicability of the derivatization procedure was tested for secondary (straight and cyclic) and tertiary alcohols. Fig. 5 shows the CPDB derivatives of *tert.*-butanol and several secondary alcohols. Interestingly, the detector response for secondary alcohols and *tert.*-butanol at the same concentrations were less than of those obtained for primary alcohols with the corresponding number of carbon atoms. However, the reaction yield obtained with CPDB were significantly higher than those obtained previously by us with the methoxylated benzofuran CPMB [18]. Similar results were also reported by others [17,19]. However, neither AQC [17] nor 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole [19] reacted with tertiary alcohols, while CPDB did react with *tert.*-butyl alcohol to give the t-butyl-CPDB ester under the same conditions utilized for the primary alcohols.

4. Conclusions

A simple synthetic procedure for the preparation of CPDB is described. Compared with current methods for the determination of alcohols, the use of

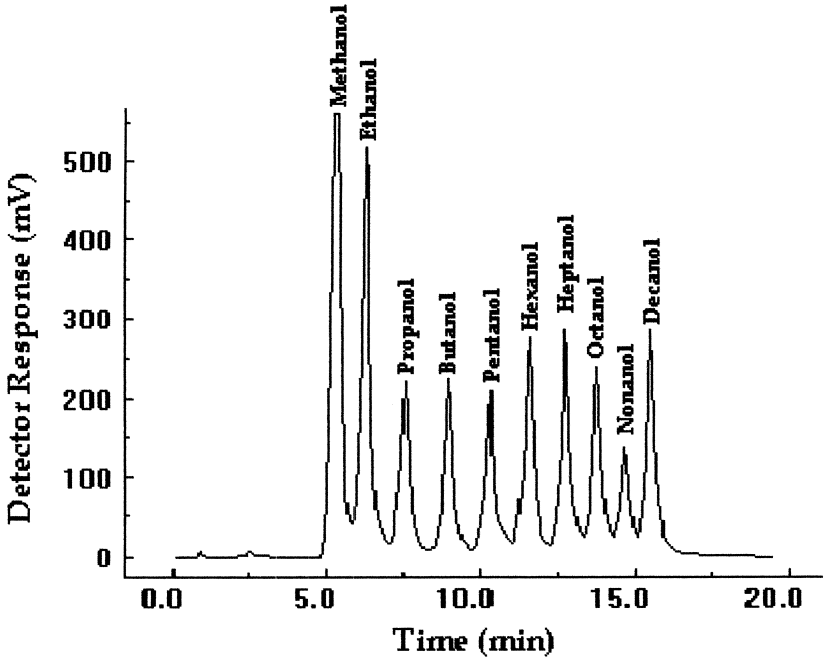

Fig. 3. Chromatogram showing the CPDB derivatives of a mixture consisting of 10 ng/ml of C_1–C_{10}-ol. Derivatization and separation were carried out as described in Section 2.

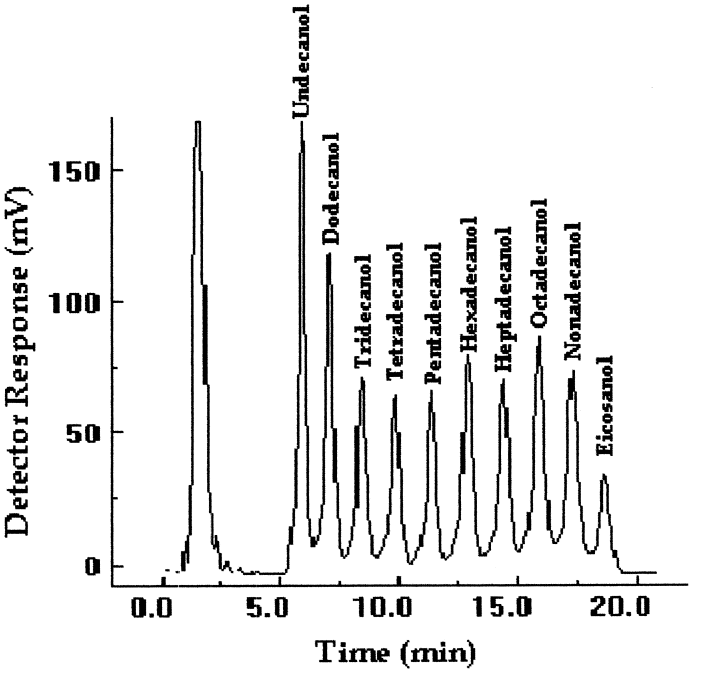

Fig. 4. Chromatogram showing the CPDB derivatives of a mixture consisting of 10 ng/ml of C_{11}–C_{20}-ol. Derivatization and separation were carried out as described in Section 2.

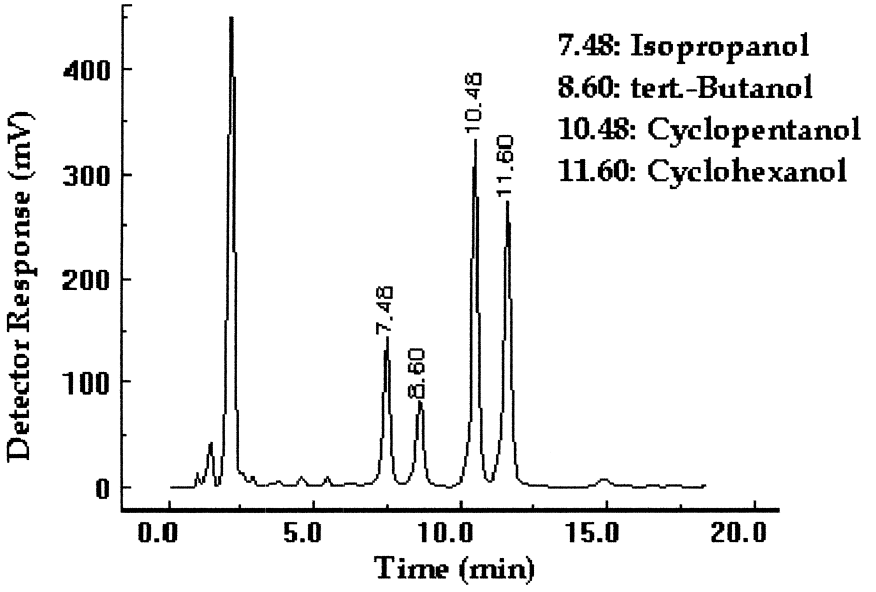

Fig. 5. Chromatogram showing the CPDB derivatives of secondary (and cyclic) and tertiary alcohols as indicated. Derivatization and separation were carried out as described in Section 2.

CPDB offers several advantages. The derivatization reaction proceeds rapidly and smoothly in aqueous solution with standard coupling agents and a base catalyst. The reagent and the derivatized alcohols are fairly stable for at least 10 days, thus facilitating automatization of the procedure. In addition, CPDB was found useful for the derivatization not only of primary alcohols, but also for secondary and tertiary alcohols. Twenty ester derivatives of CPDB were simultaneously separated within 30 min. The method is sensitive and could be easily applied for the derivatization of various hydroxyl group-containing drugs and naturally occurring compounds.

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