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Determination of alcohols by high-performance liquid chromatography with fluorimetric detection after precolumn derivatization with 2-(4-carboxyphenyl)-6-methoxybenzofuran

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Abstract

A simple procedure is described for the derivatization of primary short- and long-chain alcohols and the high-performance liquid chromatographic separation and determination of the resulting derivatives. The alcohols were derivatized to their 2-(4-carboxyphenyl)-6-methoxybenzofuran esters with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as the dehydrating agent. The coupling (esterification) reaction proceeded rapidly and smoothly in the presence of a base catalyst. An aliquot of the reaction mixture was analyzed either directly or after further purification using solid or solvent extraction procedures. The resulting esters of 30 alcohols were separated on a reversed-phase column (Ultrasphere C_8) with gradient elution and detected fluorimetrically (excitation at 315 nm, emission at 390 nm). The limits of detection (signal-to-noise ratio = 3) for the derivatized alcohols were in the range 0.1–0.5 pg per injection.

Keywords: Derivatization, LC; Alcohols; Carboxyphenylbenzofuran

1. Introduction

The hydroxyl group is a functional element of many drugs and naturally occurring compounds; however, its detection is still a challenging analytical subject. The determination of fatty alcohols and hydroxy steroids has always been a difficult analytical problem. Recently, the determination of trace amounts of fatty alcohols and dialkylglycerols from phospholipids provided valuable information about the lipid content and hence the functional properties of membranes [1–4]. Alcohols with chain lengths from 8 to 20 carbon atoms are widely used in the pharmaceutical and cosmetic industries and as raw materials in the manufacture of surfactants [5]. Consequently, the presence of alcoholic contaminants exhibiting high surface activity is a general problem in the physical pharmacy investigation of surfactant phenomena.

In addition to their determination by gas chromatography [6–9], several precolumn derivatization methods have been reported for the determination of alcohols using high-perform-

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ance liquid chromatography (HPLC). These methods involve derivatization with reagents such as phenyl isocyanate [10], 3,5-dinitrobenzyl chloride [11] and 4-naphthalene-1-azo-(4′-dimethylaminobenzene) sulfonate [12] for the determination of aliphatic alcohols with ultraviolet (UV) detection. Sensitive determinations of aliphatic alcohols and hydroxy steroids with fluorimetric detection after precolumn derivatization have also been reported. These include derivatization with 4-dimethylamino-1-naphthoyl nitrile [13], 2-methyl-1,1′-binaphthalene-2′-carbonyl nitrile [14], the azide [15] and chloride [16] derivatives of 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxaquinoxaline-2-carboxylic acid, 4-diazomethyl-7-methoxycoumarin [17], 7-methoxycoumarin-3- and -4-carbonylazides [18], 1- and 9-anthroyl nitriles [19], 1-anthracenecarboxylazide [20] and others [21]. For most of these derivatizing reagents in general, and the azides and acid chlorides in particular, the derivatization procedure involves heating in an anhydrous solvent at 100°C for 40 min and then at 130°C for 60 min [3, 4, 15, 16]. Moreover, most of the above-mentioned reagents, both in their native form and as their alcoholic derivatives, are unstable and need to be stored under cool, dry conditions.

2-(4-Carboxyphenyl)-5,6-dimethylbenzimidazole has been reported to be a useful and sensitive reagent for the determination of alcohols under mild conditions, where no anhydrous solvents or excessive heat are employed [22]. This reagent is not commercially available and experimental results from our laboratory, in terms of both synthesis and application of the method, were only partially satisfactory. Here, we report the use of 2-(4-carboxyphenyl)-6-methoxybenzofuran as a very sensitive reagent for the derivatization of simple and long-chain primary and secondary alcohols. The dependence of the derivatization procedure on the reaction conditions, the dehydrating (coupling) agent and the catalyst utilized, the separation conditions for the derivatized alcohols and the quantitative analytical characteristics of the method are 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, WI, 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), acetic acid and water were all of HPLC grade (Fischer, Santa Clara, CA, USA) and were filtered through a 4-μm filter (Rainin, Emeryville, CA, 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 2-(4-carboxyphenyl)-6-methoxybenzofuran (CPMB) 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 catalyst 4-dimethylaminopyridine (DMAP), respectively, were prepared in acetonitrile. The reagent, coupling agent and catalyst-containing solutions were stable for 1 week in daylight at room temperature.

2.2. Synthesis of 2-(4-carboxyphenyl)-6-methoxybenzofuran (CPMB)

To a solution of 3.04 g (20 mmol) of 2-hydroxy-4-methoxybenzaldehyde (4-methoxysalicylaldehyde) and 4.9 g (25 mmol) of α-bromo-*p*-toluonitrile (4-cyanobenzyl bromide) in DMF (25 ml) were added dropwise 20 ml of sodium methoxide solution [freshly prepared by dissolving 1.2 g (50 mmol) of metal sodium in dry methanol]. The resulting solution was heated at

120°C for 4 h, following which the methanol was distilled off. The condensed solution was poured into a mixture of ice-cold water (60 g) and methanol (10 ml) and stirred at 0°C for 1 h. The deposited crystals were collected, washed with water and dried in vacuo. The crude product was recrystallized from 2-propanol to give pure 6-methoxy-2-(4-cyanophenyl)benzofuran (MCPB), 3.92 g (79%); m.p., 157°C; IR (Nujol), 2228 cm^{-1} (CN); $UV_{ethanol,\lambda_{max}} = 328$ nm (log $\varepsilon = 4.54$); MS (m/z) 249 (M^+). Analysis: calculated for $C_{16}H_{11}NO_2$, C 77.09, H 4.44, N 5.26; found, C 76.27, H 4.65, N 5.54%.

A solution of MCPB (2.49 g, 10 mmol) and powdered potassium hydroxide (10 g, 180 mmol) in propylene glycol (100 ml) was refluxed for 8 h, cooled to room temperature and poured into a mixture of ice–water (100 g) and concentrated hydrochloric acid (30 ml). The deposited crystals were collected, washed with water and dried in vacuo. The crude product was dissolved in a minimum of DMF and recrystallized from ethanol to give 2.27 g (86%) of the reagent CPMB; m.p. >300°C (decomp.); IR (Nujol), 1690 cm^{-1} (C=O); $UV_{ethanol,\lambda_{max}} = 315$ nm (log $\varepsilon = 4.51$); MS (m/z) 268 (M^+). Analysis: calculated for $C_{16}H_{12}O_4$, C 71.63, H 4.51; found, C 71.75, H 4.56%.

2.3. Isolation of dodecyl-CPMB

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, Sunnyvale, CA, USA). The cartridge was washed three times each with 3 ml of 50% methanol in water to remove excess reagents. The dodecyl-CPMB 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 >97%), which 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 Shimadzu LC 600 pump and a Shimadzu RF 535 fluorescence detector operating at excitation and emission wavelengths of 315 and 390 nm, respectively. Samples were injected manually using a Rheodyne 20-μl sample loop (Rainin, Emeryville, CA, USA). Ultraviolet spectra were recorded with a Model 8425 A diode-array UV spectrophotometer (Hewlett-Packard, Palo Alto, CA, USA) and fluorescence was recorded with a DM 3000 spectrofluorometer (Spex Industries, Edison, NJ, USA).

2.5. Chromatographic conditions

The HPLC apparatus was connected to an Ultrasphere C_8 column (150×4.6 mm I.D., 5 μm) (Beckman, Palo Alto, CA, USA). For separation of a mixture of C_1–C_{20}-ol derivatives, the samples were eluted at ambient temperature with 35% acetonitrile in water containing (A) 0.1% acetic acid and (B) 0.1% acetic acid in pure acetonitrile. Gradient elution from 30% to 100% B in 20 min was used at a flow-rate of 1 ml/min.

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 methanol to eicosanol, 5 ng/ml final concentration) were added 2 ml each of DMAP, EDC and the reagent CPMB in 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 into the chromatograph either directly or after one of the following purification steps: 2- and

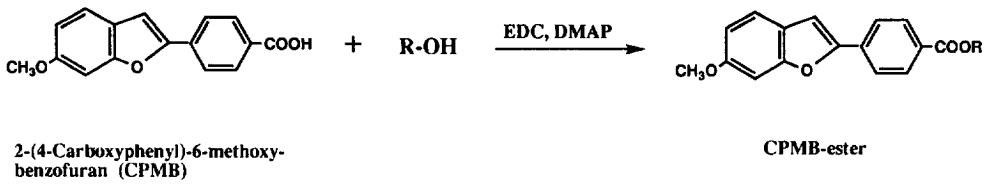

Fig. 1. Scheme for the derivatization reaction of CPMB with alcohols.

5-ml volumes of 0.1 *M* aqueous NaOH and *n*-hexane, respectively, were added and the tube was vortex mixed for 1 min. The layers were separated by centrifugation at 1000 *g* for 2 min and the organic layer was collected and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 2 ml of mobile phase B, 10 μl of which were injected into the chromatograph. Alternatively, while hot (60°C), the acetonitrile in the reaction mixture was evaporated to dryness under a stream of nitrogen and the residue treated by the same procedure as described above for the isolation of dodecyl-CPMB ester using a C_{18} cartridge.

2.7. Recovery, accuracy, precision, linearity and reproducibility studies

In these experiments, known amounts of alcohol (1-octanol) were added to phosphate buffer (pH 7.4) and extracted three times with four volumes each of *n*-hexane. The combined hexane extracts were evaporated to dryness under a stream of nitrogen. The residue was reconstituted with acetonitrile and derivatized according to the procedure described above. At the end of the derivatization, dodecyl-CPMB was added as an internal standard. A calibration graph (peak-height ratio versus concentration) was constructed by injecting 100-μl volumes of solutions containing known amounts of preformed octyl-CPMB derivative equivalent to 5, 10, 20, 40, 80 and 100 ng/ml of the native alcohol (1-octanol) containing the internal standard dodecyl-CPMB. Accuracy and recovery were determined from values obtained following actual analysis of the native alcohol as calculated from the calibration graph constructed by using the preformed alcoholic derivatives.

3. Results and discussion

3.1. Derivatization conditions

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

Solvent effect

Acetone, acetonitrile, benzene, chloroform, dichloromethane, dioxane, DMF, dimethyl sulfoxide, ethylene dichloride, ethyl acetate and pyridine were tested for their suitability as reaction solvents for the derivatization procedure. Table 1 shows that acetonitrile and ethyl acetate gave the best results as assessed by the detector response. However, acetonitrile was used as the reaction solvent throughout this study because of the sparing solubility of EDC in ethyl acetate and the immiscibility of ethyl acetate in the mobile phase.

Base cataylst

No reaction took place without a base catalyst. Table 2 shows that among the various bases tested, 4-diemthylaminopyridine (4%) and 4-pyrrolidinopyridine (10%) gave the highest detector responses. Several concentrations of DMAP were tested. Concentrations in excess of 4% did not increase the reaction yield and were

Table 1
Effect of various derivatization reaction solvents on detector response (reaction yield)

Solvent	Detector response (%)	Solvent	Detector response (%)
Acetone	27	Dioxane	20
Acetonitrile	100	N,N-Dimethylformamide	10
Benzene	32	Dimethyl sulfoxide	10
Chloroform	80	Ethylene dichloride	90
Dichloromethane	85	Ethyl acetate	100

Reactions were carried out at 60°C for 30 min with 100 ng/ml of dodecyl alcohol, EDC and DMAP at 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%.

Table 2
Effect of base catalyst on yield of the derivatization reaction

Base catalyst	Detector response (%)
4-Dimethylaminopyridine (4%)	100
4-Pyrrolidinopyridine (10%)	100
Pyridine (10%)	5
Triethylamine (10%)	8
Tributylamine (10%)	10

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%.

accompanied by unidentified interfering peaks and therefore were avoided. Concentrations of less than 3% DMAP led to sub-maximum responses. The use of 10% solutions of 4-pyrrolidinopyridine (more expensive) did not offer any advantage over 4% of DMAP.

Carbodiimide and other coupling agents

Table 3 shows the detector response obtained when various coupling agents were tested in the derivatization reaction. Amongst these, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) gave the best detector response and was therefore 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.

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 completed within 10, 30 and ca. 45 min at 100, 80 and 60°C, respectively. Uniden-

Table 3
Effect of the coupling agent on the derivatization reaction yield

Coupling agent	Detector response (%)
Dicyclohexylcarbodiimide	7
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)	100
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide methiodide	15
1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate	20
2-Isobutoxyl-1-isobutoxycarbonyl-1,2-dihydroquinoline (IIDQ)	10
1-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ)	24
1-Hydroxybenzotriazole	18

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 a 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%.

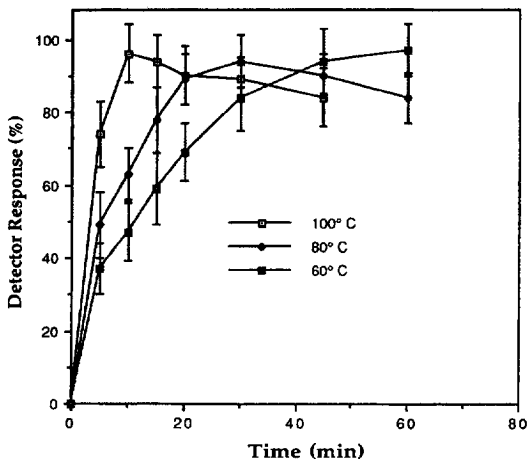

Fig. 2. Dependence of the detector response and the time of completion of the derivatization reaction on temperature. Reactions were carried out at the specified temperature with 100 ng/ml of dodecyl alcohol in the presence of CPMB, EDC and DMAP at final concentrations of 0.01, 2 and 4%, respectively.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

tified 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 maximum response. Increasing the reagent concentration to more than 0.01% (already a large excess of reagent to alcohol) did not significantly alter the time and temperature needed for the derivatization reaction to be completed.

3.2. Separation and determination of CPMB derivatives

Fluorescence measurements yielded excitation and emission maxima for dodecyl-CPMB at 310 and 390 nm, respectively. When other derivatives were tested (i.e., C_8–C_{16}-ol), similar maximum 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, 2-propanol, DMF, tetrahydrofuran and acetonitrile in pure form and in aqueous mixture combinations. Several normal- and reversed-phase columns were tested (silica, C_4, C_8, C_{18}, C_{18}-cyano and C_{18}-amino). For the 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 all twenty alcohols were separated within 30 min (Fig. 3). Acetic acid, at a concentration of 0.1%, was found to be the best organic modifier and gave the sharpest peaks.

3.3. Secondary and tertiary alcohols

The applicability of the derivatization procedure was tested for secondary (straight-chain and cyclic) and tertiary alcohols. Fig. 4 shows the CPMB derivatives of *tert.*-butanol and several secondary alcohols as obtained (A) before and (B) after the purification procedure using a C_{18} cartridge as described above. Interestingly, the detector responses for secondary alcohols and *tert.*-butanol at the same concentrations were less than half of those obtained for the primary alcohols with the corresponding number of carbon atoms. Similar results were also reported by Katayama et al. [22]. However, with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole as the reagent, Katayama et al. [22] reported no reaction with tertiary alcohols, whereas CPMB did react with *tert.*-butyl alcohol to give the *tert.*-butyl-CPMB ester under the same conditions as utilized for the derivatization of primary alcohols. No apparent explanation for this difference in esterification reactivity of 2-substituted benzofuran and benzimidazole is available.

Fig. 5 demonstrates the application of this method (A) for a mixture of ethanol, butanol and hexanol and (B) for the determination of alcoholic contaminants present in a commercial sodium dodecyl sulfate preparation.

3.4. Quantitative analytical characteristics

The precision and reproducibility of the method were demonstrated by the determination of

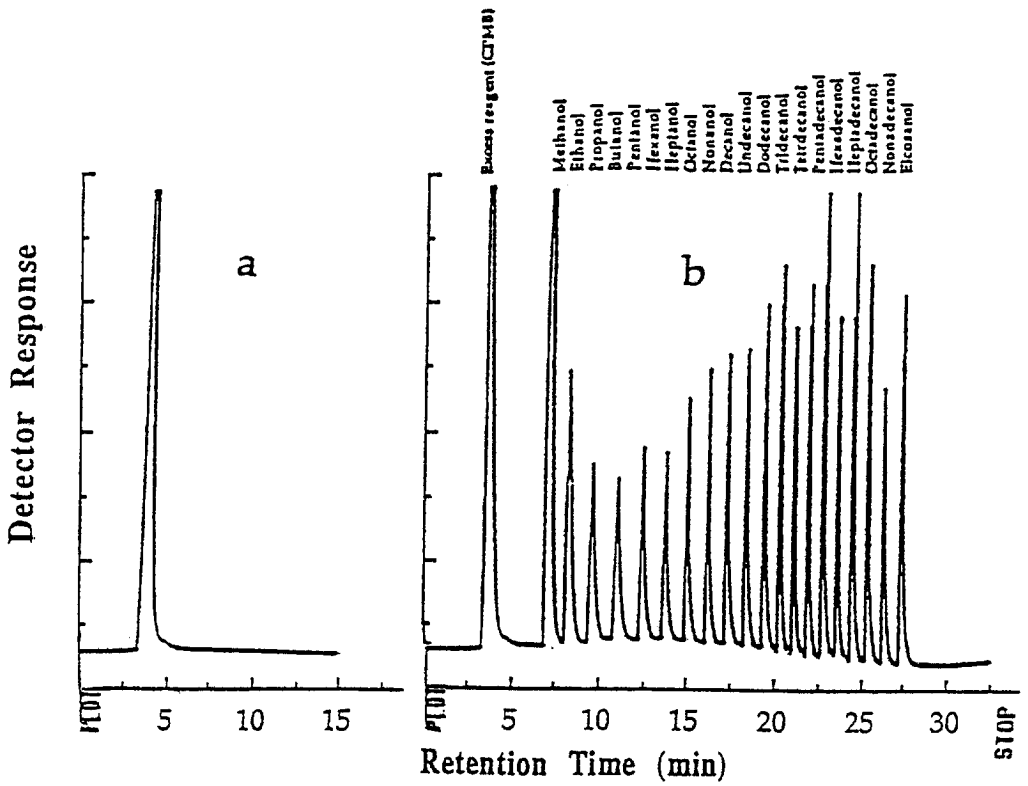

Fig. 3. Chromatogram of (a) the reagent blank and (b) CPMB-esters of C_1–C_{20}-ol (methanol–eicosanol). A mixture consisting of 5 ng each of the alcohols was derivatized with CPMB and separated according to the procedure described in the text.

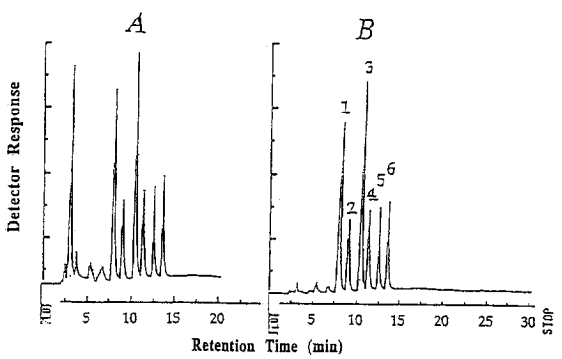

Fig. 4. Chromatogram of CPMB esters of (1) 2-propanol, (2) *tert.*-butanol, (3) 2-pentanol, (4) cyclohexanol, (5) cycloheptanol and (6) cyclooctanol. A mixture consisting of 10 ng each of the alcohols was derivatized and injected (A) before and (B) after purification on a C_{18} cartridge according to the procedure described in the text.

unknown concentrations of 1-octanol in quintuplicate on three separate (consecutive) days. The mean (±S.D.) data of each analysis day are presented in Table 4. The accuracy of the method ranges from 0.57 to 8.8% (median 3.29%) with high precision and reproducibility (expressed as mean percentage rather than as range or average deviation). The accuracy range is probably due to the incomplete recovery of the alcohol upon hexane extraction rather than to non-quantitative conversion of the extracted alcohol to its CPMB-ester derivative. This conclusion is based on the finding that direct derivatization of 1-octanol dissolved in acetonitrile to the same concentrations (thus precluding the hexane extraction step) yielded accuracy in the range 0.37–1.03%. The sensitivity of the method is demonstrated in Fig. 5A, which shows the chromatogram obtained following injection of a

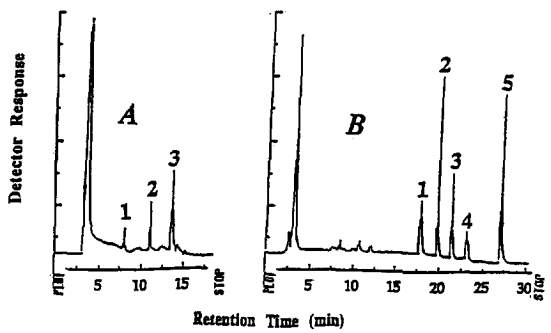

Fig. 5. (A) Chromatogram obtained following injection of a 100-μl volume of a derivatization reaction mixture containing 0.5, 1 and 2 pg per injection of (1) ethanol, (2) butanol and (3) hexanol, respectively. (B) Chromatogram showing the presence of (1) decanol, (2) dodecanol, (3) tetradecanol and (4) hexadecanol as alcoholic contaminants in a commercial preparation of sodium dodecyl sulfate (SDS). Eicosanol-CPMB (5) was used as an internal standard. The derivatization reaction was carried out as described in the text with an acetonitrile-reconstituted residue following hexane extraction of 1 ml of SDS aqueous solution containing 0.3 mg/ml.

100-μl volume of a derivatization mixture containing 5, 10 and 20 pg/ml of ethanol, butanol and hexanol, respectively. Assuming an appropriate chromatographic peak volume of 0.2 ml, the minimum detectable amount for the method is 0.1 pg or lower. The method appears to be linear over a wide range of alcohol concentrations and a typical calibration graph of the peak-height ratio (y) of derivatized 1-octanol to dodecyl-CPMB (internal standard) versus 1-octanol concentration (x) was $y = 0.02x + 0.012$ ($r^2 = 0.994$). Regression line plots of actual versus theoretical concentrations for the data in Table 4 do not deviate from the line of identity, indicating no method bias.

4. Conclusions

Compared with currently available methods for the determination of alcohols, the proposed method offers a number of advantages: the derivatizing reagent is easily synthesized from commercially available precursors; anhydrous reaction conditions are not required for the derivatization procedure or the storage of the native reagent or the derivatized samples; the method is facile, inexpensive, sensitive, reproducible and utilizes simple derivatization conditions, yielding a complete reaction within 1 h at 60°C;

Table 4
Recovery, accuracy and precision values derived from mean (±S.D.) values of concentrations of 1-octanol actually determined in quintuplicate analyses as calculated from the calibration graph constructed from preformed CPMB derivatives (theoretical) on three separate days

Theoretical concentration (ng/ml)	Actual (observed) concentration (ng/ml)					
	Day 1	R.S.D. (%)	Day 2	R.S.D. (%)	Day 3	R.S.D. (%)
5	4.69 ± 0.13	2.7	4.57 ± 0.11	2.4	4.46 ± 0.21	4.7
10	9.44 ± 0.32	3.4	9.61 ± 0.41	4.3	9.37 ± 0.52	5.5
20	19.1 ± 0.7	3.8	19.3 ± 0.8	4.2	18.8 ± 1.1	5.6
40	38.7 ± 1.6	4.2	38.2 ± 1.6	4.1	39.3 ± 1.3	3.3
60	57.4 ± 2.0	3.6	56.9 ± 3.2	5.5	58.2 ± 2.9	4.9
80	77.0 ± 3.4	4.5	75.9 ± 4.2	5.4	75.2 ± 4.6	6.1
100	96.5 ± 4.2	4.3	95.5 ± 6.3	6.6	95.0 ± 4.7	4.9
Recovery (%)[a]	95.5 ± 4.2		95.2 ± 4.7		94.3 ± 5.4	
Accuracy (%)[b]	0.65–8.8		0.57–7.4		0.86–7.4	
Precision and reproducibility[c]	–		99.4%		98.7%	

[a] Calculated as mean ±S.D. of all concentrations.
[b] Expressed as the range of % error of the individual values.
[c] Calculated as the average of the mean individual recovery values compared with day 1 of analysis.

unlike the non-selectivity of azides or acyl chloride derivatives, where reaction of the reagent with amines and amino acids is inevitable, the carboxylic function of CPBM is relatively selective for alcohols under the conditions described; and the method may be used with either reversed- or normal-phase systems. Because of the stability of the benzofuranyl ester adducts, the method can be easily applied for routine analyses of large numbers of samples using automated systems. The chromatographic conditions are simple, do not necessitate the use of complex buffered solutions, ion-pairing or specific organic modifiers and yet facilitate the separation and complete resolution of 20 or more alcohols in the same run within a relatively short time. Finally, the method can be generalized for the determination of secondary and tertiary alcohols and other hydroxy-bearing compounds. In fact, preliminary results indicate the suitability of the method for application to hydroxy steroids by HPLC with fluorimetric detection. The scope of utilization of the method is currently under active investigation.

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