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Determination of alcohols by high-performance liquid chromatography with fluorimetric detection after pre-column derivatisation with carbazole-9-*N*-acetic acid and chromatographic behaviour of alcoholic derivatives

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Abstract

Alcohols were derivatised to their carbazole-9-*N*-acetic acid (CRA) esters with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) as the dehydrating agent. Studies on derivatisation conditions indicated that the coupling reaction proceeded rapidly and smoothly in the presence of a base catalyst in acetonitrile to give the corresponding sensitively fluorescent derivatives. The retention behaviour of alcohol derivatives was investigated by varying mobile phase compositions (ACN–water and MeOH–water). The parameters from the equation $\log k' = A - BX$ were evaluated by retention data of derivatives using an isocratic elution with different mobile phases. The results indicated that the parameters derived allowed computation of retention factors in good agreement with experiments. At the same time, a general equation was derived that makes possible predictions of partition coefficient in binary mobile phases with different proportions of organic solvent to water based on some simple regression analysis. The LC separation for the derivatised alcohols containing higher carbon alcohols showed good reproducibility on a reversed-phase C_{18} column with gradient elution. The detection limits (excitation at 335 nm, emission at 360 nm) for derivatised alcohols (signal-to-noise ratio=3:1) were in the range of 0.1–0.4 pg per injection.

Keywords: Derivatisation, LC; Alcohols; Carbazole-9-*N*-acetic acid

1. Introduction

Alcohols are widely used in the pharmaceutical and cosmetic industries and as raw materials in the manufacture of surfactant [1]. However, the main difficulty with the liquid chromatography (LC) of these substances is their detection. Many methods including gas chromatography [2,3], enzymatic [4,5] and spectrophotometric methods [6] have been described for the determination of alcohols. Derivatisation of the hydroxyl groups has often been undertaken in order to increase the sensitivity of the method. Several pre-column derivatisation methods have been reported for the determination of alcohols using high-performance liquid chromatography (HPLC). These methods involve derivatisation with

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reagents such as phenyl isocyanate [7], 3,5-dinitronbenzyl chloride [8] and 4-naphthalene-1-azo-(4′-dimethylaminobenzene)-sulfonate [9] for the determination of aliphatic alcohols with ultraviolet (UV) detection. Sensitive determinations of aliphatic alcohols and hydroxyl steroids with fluorimetic detection after pre-column derivatisation have also been reported. These include derivatisation with 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one (Br-MBX) [10], 4-dimethylamino-1-naphthoyl-nitrile [11], 2-methyl-1′,1′-binaphthalene-2′-carbonyl nitrile [12], the azide [13] and chloride [14] derivatives of 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxaquinoxaline-2-carboxylic acid, 4-diazomethyl-7-methoxycoumarin [15], 7-methoxy-coumarin-3- and -4-carbonylazides [16], 1- and 9-anthroyl nitriles [17], 1-anthracenecarboxylazide [18,19]. In addition, esterfication of alcohols with carboxylic acids in the presence of *N*,*N*′-carbonyldiimidazole under mild conditions has also been reported [20]. Although this reaction gives high ester yield in the presence of alkaline catalysts such as metal sodium or sodium alcoholate. However, it is not a convenient procedure because of the use of sodium metal or sodium alcoholate catalyst. Recently, a report on the preparation of 9-fluoreneacetic acid and 4-biphenylacetic acid derivatives of dihydroqinghaosu (DQHS) using 2,4,6-trimethylbenzene-sulfonyl chloride (METS-chloride) and 2,4,6-triisopropylbenzene-sulfonyl chloride (TIPS-chloride) as condensation regents, respectively, has appeared [21]. They are very convenient procedures superior to previous approaches that require prior conversion of carboxylic acid to acid chloride or acyl nitrile or acylazide or acid anhydride. However, the condensation reagents METS-chloride and TIPS-chloride are the hindered benzenesulfonyl chlorides, low reactivities are observed probably due to introducing three substituting groups in a benzene nucleus. For most of these derivatising reagents mentioned above in general, the azides and anhydrides in particular, the derivatisation procedure involves heating in an anhydrous solvent at 100°C for 40 min and then at 130°C for 60 min [13,14,22,23]. Moreover, both in their native form and as their alcoholic derivatives are unstable and need to be stored under cool, dry conditions. Acid chlorides, in particular, are neither satisfactory nor convenient procedures because of their instability towards moisture.

In a previous paper [24], we have already described the synthesis and analytical application of carbazole-9-*N*-acetyl acid (CRA) for the determination of amino acids after it is converted to corresponding acid chlorides. Recently, we have also reported the preparation of an activated anhydride of carbazole-9-*N*-(2-methyl)-acetyl-benzene-disulfonate (CMABS), which can rapidly label alcohols including volatile alcohols from human plasma in anhydrous solvent in the presence of triethylamine or pyridine catalyst [25]. But derivatisation of the activated anhydride with alcohols is only limited to C_1–C_9 under the HPLC conditions proposed. In this study, the principal goal is to use parent CRA molecule to react directly with hydroxyl compounds in the presence of a dehydrating agent and a base catalyst, where no anhydrous solvents or excessive heat are employed. The method can easily label long-chain primary alcohols under mild conditions. In addition, an equation was developed to predict the retention factor, k'; of the CRA derivatives in the present HPLC mode.

2. Experimental

2.1. Instrumentation

A Model 655 liquid chromatograph equipped with a Model 650-10S spectrofluorimeter (Hitachi Seisakusho, Tokyo, Japan), a Model 7125 injection valve (Rheodyne, Cotati, CA, USA), a Model 655 proportioning valve and a Model 644-61 integrator (Hitachi Seisakusho) were used. Fluorescence excitation and emission spectra were also obtained on a Model 650-10S spectrofluorimeter. Excitation and emission bandpass are both at 15 nm. Alcohol derivatives were separated on a 200×4.6 mm I.D. 5 μm Spherisorb C_{18} (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China). A Paratherm U_2 electronic water-bath (Hitachi, Tokyo, Japan) was used to control column temperature. The mobile phases were treated ultrasonically for 15 min in order to remove gas bubbles prior to use.

2.2. Chemicals

Triethylamine, pyridine, 2-methylpyridine, tri-

butylamine and 4-dimethylaminopyridine were treated with molecular sieve and potassium hydroxide pellets, respectively, then was redistilled prior to use. Double distilled water was used throughout. Dichloromethane, chloroform, acetone and other reagents were of analytical-reagent grade. Acetonitrile (ACN) was chromatographic grade. All alcohols used in experiments were from Shanghai Chemical Reagent (Shanghai, China). Carbazole-9-*N*-acetyl acid was prepared according to the method we previously described [24].

Standard solutions (100 ng/ml) of the alcohols from C_1–C_{15} were prepared by dilution with acetonitrile of stock solutions of each alcohol (1 mg/ml) in acetonitrile. The reagent solution $4.5 \cdot 10^{-5}$ mol/l was prepared by dissolving 1 mg CRA in 2 ml acetonitrile and diluting to 100 ml with acetonitrile. Solutions of $5.0 \cdot 10^{-5}$ mol/l and 0.1 mol/l coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC·HCl) and base catalyst 4-dimethylaminopyridine (DMAP) were prepared in acetonitrile, respectively. Commercial potassium salt of alkyl phosphate ester was supplied by Chemical Factory, Qufu Normal University, Department of Chemistry (Qufu, Shandong, China). Plasma samples were obtained from Qufu Traditional Chinese Medical Hospital (Qufu, Shandong, China).

2.3. Chromatographic method

Isocratic elution was adopted for the evaluation of chromatographic behaviour of alcoholic derivatives. LC separation of alcoholic derivatives was performed on a Spherisorb C_{18} column with a binary gradient. Eluent A was 0.1% acetic acid+0.1% triethylamine (pH 6.5)–acetonitrile (65:35, v/v) and B was acetonitrile–water (95:5, v/v). The flow-rate was constant at 1.0 ml/min and the column temperature was kept at 30°C. The gradient conditions used for the separation of alcoholic derivatives have been indicated in the legend of each chromatogram.

2.4. Derivatisation procedure

The derivatisation reaction is shown in Fig. 1. To 200 μl a standard solution of the alcohols in a tube (each alcohol from methanol to 1-pentadecanol, 10 ng/ml final concentration) were successively added 50 μl each of DMAP, EDC and the reagent CRA,

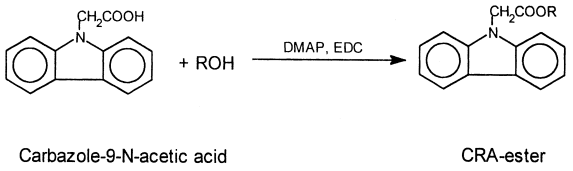

Fig. 1. Scheme for the derivatisation reaction of CRA with alcohols.

respectively. The tube was then sealed and the mixture was heated at 60°C for 30 min in a thermostatic water-bath 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 the following purification step: To the reaction mixture mentioned above was added a 5-ml volume of *n*-hexane. The mixture was washed successively with 4 ml each of 1 *M* hydrochloric acid, water, 1 *M* sodium hydroxide and deionized water, respectively. The organic phase was separated and evaporated to dryness under a stream of nitrogen. Residue was redissolved in 100 μl mobile phase B, 10 μl of which was injected into the chromatograph.

2.5. Alcohols extracted from potassium salt of alkyl phosphate ester

An application for the analysis of higher carbon alcohols extracted from a commercial potassium salt of alkyl phosphate ester, in conjunction with a gradient elution, was investigated. The following pretreatment was adopted in order to extract the alcohols before the LC analysis. To 3.0 g potassium salt of alkyl phosphate ester was added 10-ml of deionized water, the mixture was stirred at room temperature for 15 min and then 4- and 5-ml volumes of 0.1 *M* aqueous NaOH and *n*-hexane were added, respectively. The final mixture was vortex-mixed for 10 min. The organic layer was collected. The residue was again extracted with another 5-ml *n*-hexane. The combined hexane extract was washed successively with 4 ml each of 1 *M* hydrochloric acid, 1.0% sodium hydrogencarbonate and deionized water, respectively. The organic layer was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 2 ml acetonitrile and derivatised as described in Section 2.4. A 10-μl

volume of which was injected into the chromatograph.

2.6. Preparation for plasma sample

(1) Plasma sample without further treatment: a 0.1-ml portion of the plasma sample solution (after centrifugation) was directly pipetted into a 10-ml glass tube for the derivatisation.

(2) The extraction procedure of plasma and the optimisation of the extraction procedure were previously described in our study [26].

3. Results and discussion

3.1. Solvent effect

Acetonitrile, dichloromethane, ethyl acetate, chloroform and acetone were investigated as reaction co-solvents for derivatisation procedure. The reactions were carried out at 60°C for 30 min with 50 ng/ml of *n*-butanol, $4.5 \cdot 10^{-5}$ mol/l of EDC·HCl and 0.1 mol/l of DMAP, respectively. The results indicated that acetonitrile and ethyl acetate gave the best results as assessed by the detector response. A slight decrease in detector response in chloroform (90%) and dichloromethane solvents (85%) was observed. Acetone gave the lowest response (32%) under the conditions proposed. In general, acetonitrile was used as the reaction solvent throughout this study because of the sparing solubility of EDC·HCl in ethyl acetate and the immiscibility of ethyl acetate in the mobile phase.

3.2. Base catalyst

Triethylamine, pyridine, DMAP, 2-methylpyridine and tributylamine are investigated as reaction catalysts. Reactions were carried out at 60°C for 30 min with 50 ng/ml of *n*-butanol using EDC·HCl as the coupling agent ($4.5 \cdot 10^{-5}$ mol/l) in the presence of various base catalyst (concentration 0.1–0.2 mol/l). Each value is an average of six runs with the detector response obtained with DMAP taken as 100%. It is found that DMAP gives the highest detector responses. Most subsequent derivatisation is carried out by the use of DMAP as the base catalyst. Further study indicates that the final DMAP concentration in derivatised solution should be >0.1 mol/l to give complete derivatisation, with further increasing the concentration of DMAP in derivatised solution does not significantly increase the reaction yield.

3.3. The effects of different coupling agents on detector response

Dicyclohexylcarbodiimide (DCC) and EDC·HCl as reaction catalysts were investigated. Reactions were carried out at 60°C for 30 min with 50 ng/ml of *n*-butanol using DMAP (0.1 mol/l) as the base catalyst in the presence of $4.5 \cdot 10^{-5}$ mol/l EDC·HCl and DCC, respectively. Detector response for derivatising *n*-butanol using EDC·HCl as coupling agent was eight times greater than that of DCC under the derivatisation conditions proposed. EDC·HCl proved optimal as reaction condensation agent as it was freely soluble in acetonitrile [27] or dissolved in water [28]. It was also observed that if coupling reagent was insufficient to obtain maximal yield, addition of more coupling reagent could reproducibly increase the yield to the maximum. In general, the % yield of the derivatisation procedure for an unknown concentration sample was calculated by integrating the peak areas reached maximum for the derivatised solutes by addition of increasing amounts of EDC·HCl. In most cases, $4.5 \cdot 10^{-5}$ mol/l EDC·HCl was used for the derivatisation of the analytes.

3.4. Temperature conditions and time effects

The optimum temperature and time for derivatisation were investigated. The results indicated that heat had a significant effect on 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. It was found that above 80°C the position of the equilibrium reduced the proportion of CRA reacting with alcohols because of forming some by-products (unidentified); while below 60°C the rate of reaction was decreased and led to long derivatisation time. Although, a clean reaction occurred at room temperature, times in excess of 24 h or more were needed for a maximum response.

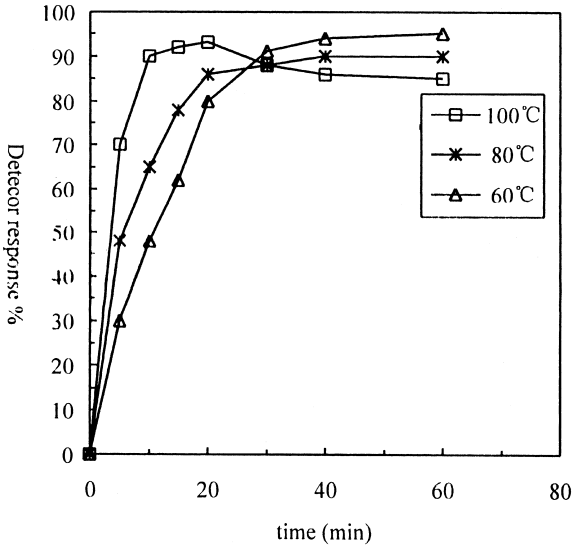

Fig. 2. Dependence of detector response and time of completion of the derivatisation reaction on temperature. Reactions were carried out at the specified temperature with 100 ng/ml of 1-octanol in the presence of CRA, EDC and DMAP, at concentrations of $4.5 \cdot 10^{-5}$, $5.0 \cdot 10^{-5}$ and 0.1 mol/l, respectively.

Therefore, most subsequent derivatisation temperature selected in experiments was 60°C. Increasing the reagent concentration to more than $4.5 \cdot 10^{-5}$ mol/l (already a large excess of reagent to alcohol) did not significantly alter the time and temperature needed for derivatisation reaction to be completed.

3.5. Chromatographic behaviour of straight chain alcoholic derivatives

A systematic investigation of a series of homologous alcohols was undertaken under different mobile phase compositions. In this study, by varying the volume percentage of acetonitrile or methanol, X, in the eluent for each mono-alcohol derivative of the series H $(CH_2)_n$OH, a linear relationship between the logarithm of the retention factor k' and the volume percentage, X, was observed, the dependence of the retention factor on the volume percentage, X, can be expressed by the relationship [29]:

$$\log k' = A - BX \quad (1)$$

The linear regression data between log k' and organic modifier concentration (X) according to Eq. (1) were obtained and shown in Table 1. For each alcohol which the values of n_c between 1 and 7, plots are linear with correlation coefficients $\gamma^2 \geq 0.998$. It is worth noting that a more general quadratic equation must usually be applied for analysis the dependence of log k' vs. X for a wide variation of X [29,30]; in this study a simple first-order equation, Eq. (1), represents the observed behaviour for the practical k' values.

In a homologous series the increase in the length of the aliphatic chain causes a non-specific increase in retention which may be distinguished from the contribution of the specific terms given by the interaction of the functional group of the series with mobile and stationary phases [31]. Here, a linear relationship has also been observed between log k' and the number of carbon atoms n_c of a homologous series of alcohols derivatised with CRA under each isocratic condition employed, according to the following relationship:

$$\log k' = \log \beta + \log \alpha n_c \quad (2)$$

The linear regression data for alcohol derivatives

Table 1
The results of the linear regression between log k' values and organic modifier concentrations (X) according to the equation $\log k' = A - BX$

Carbon numbers	ACN–water			MeOH–water		
	A	B	γ^2	A	B	γ^2
1	1.2653	2.0638	0.9989	2.2743	3.0677	0.9993
2	1.5834	2.3897	0.9993	2.4582	3.1383	0.9991
3	1.9067	2.6789	0.9995	2.7502	3.3591	0.9995
4	2.2501	2.9935	0.9998	3.1671	3.6287	0.9999
5	2.5777	3.2377	0.9999	3.3570	3.7165	0.9998
6	2.9253	3.5580	0.9996	3.5588	3.8276	0.9999
7	3.3400	3.9317	0.9989	3.8369	3.9204	0.9991

Table 2
Linear regression data between the log k' and the carbon atom numbers n_c at various mobile phase compositions

Mobile phase	Concentration (X) (%)	Log B	Log α	γ^2
ACN–water	45	0.1169	0.2005	0.9989
	50	0.0081	0.1926	0.9981
	55	−0.0853	0.1764	0.9983
	60	−0.1504	0.1563	0.9983
	65	−0.2741	0.1471	0.9991
	70	−0.3865	0.1386	0.9993
MeOH–water	50	0.5727	0.1971	0.9944
	55	0.3764	0.1846	0.9944
	60	0.2328	0.1774	0.9966
	65	0.1055	0.1656	0.9974
	70	0.0456	0.1588	0.9975
	80	−0.3224	0.1435	0.9875

between the log k' and the carbon numbers n_c according to Eq. (2) are shown in Table 2. For such plots the intercept log β represents the specific contribution to the retention by hydroxyl group residue for each eluent, while the slope represents the non-specific contribution to the retention by the –(CH_2)– unit. It can be seen from Table 2 that log β, which represents the specific selectivity within the investigated series, are systematically higher for methanol–water than for acetonitrile–water eluents. The parameter log β is very important with regard to selectivity, and its value generally increases with increasing the polarity of the organic modifier. It is interesting to note that little difference for logα values in both mobile phase compositions are observed. This is probably due to the fact that log α value represents the non-specific contribution, which supplied by the same CH_2 unit. It has been shown elsewhere [29–31] that linear dependence of log α and log β on the concentration of the organic solvent in the mobile phase, X and of A and B on the number of methylene groups in the aliphatic chain of each series are generally observed. According to equations similar to the ones reported in Eqs. (1) and (2), the following equations are expressed:

$$\log \beta = \beta_0 - \beta_1 X \quad (3)$$

$$\log \alpha = \alpha_0 - \alpha_1 X \quad (4)$$

$$A = a_0 + a_1 n_c \quad (5)$$

$$B = b_0 + b_1 n_c \quad (6)$$

The relationship between B and A is expressed by following equation:

$$B = q + pA \quad (7)$$

All the parameters, derived by linear regression analysis of such plots, are collected in Table 3. After the evaluation of the chromatographic behaviour of the homologous series family $H(CH_2)_nOH$ derivatised with CRA through parameters, α, β, A and B, the following Eq. (8) has been used to predict retention values for a homologous alcohols for the different mobile phase compositions:

$$\log k' = (a_0 + a_1 n_c) \cdot (1 - pX) - qX \quad (8)$$

The parameters to be inserted in Eq. (8) are those obtained from the regression data of Eqs. (3)–(7).

Table 3
The regression parameters β_0, β_1, α_0, α_1, b_0, b_1, a_0, a_1, q and p in various mobile phase compositions for homologous family $H(CH_2)_nOH$ derivatised with CRA and eluted on a Spherisorb C_{18} (5 μm) column (all regression coefficients >0.995)

System	β_0	β_1	α_0	α_1	b_0	b_1	a_0	a_1	q	p
ACN–water	0.998	1.959	0.322	0.266	1.765	0.304	0.895	0.342	0.971	0.887
MeOH–water	1.877	2.711	0.283	0.177	2.909	0.153	1.986	0.268	1.759	0.576

The comparison between experimental k' values and those computed with Eq. (8) shows good agreement (see Table 4, here alcohols investigated from C_1 to C_7). The parameters p, a_1 and b_1 remain almost constant even for different columns and for different homologous series, they represent the non-specific contribution to retention from regular increase of the alkyl chain. This has been verified by other previous work [32]. However, it is interesting to note that these parameter values increase from acetonitrile–water to methanol–water mobile phases probably due to the lower polarity of ACN resulting in a levelling effect on the behaviour of the various solutes. The parameter q, a_0 and b_0 values which represent the specific contribution linked to the nature of the functional group increase also from acetonitrile–water to methanol–water mobile phases and vary widely. In fact, alcohols, which the values of n_c between 8 and 10, plots are also linear within the ranges $0.5<\log k'<1.5$ and $75\%<X<95\%$ (v/v), with correlation coefficients $\gamma^2 \geq 0.997$, data not shown. The retention behaviour of further higher carbon alcohols are not tested because they are difficult for the simultaneous elution under an isocratic conditions especially for lower mobile phase compositions.

3.6. Analytical performance

The calibration graph was carried out by injecting 10-μl volumes of solutions containing known amounts of 1-dodecanol-CRA derivative equivalent to 5, 10, 20, 40, 80 and 100 ng/ml of 1-dodecanol containing the internal standard 1-octanol-CRA. The calibration graph was established with the peak-height ratio (y) of derivatised 1-dodecanol to 1-octanol-CRA (internal standard) versus 1-dodecanol concentration (x), the linear regression equation obtained was $y=0.0364+0.0198x$ ($n=6$, $\gamma^2=0.996$). The precision and reproducibility of the proposed method are investigated by the analysis of 1-dodecanol at 10, 40 and 100 ng/ml. The results are shown in Table 5. The analytical results of intra- and inter-day at the three concentrations are all below 3.0%, indicating satisfactory precision of the method proposed. Extraction recovery was evaluated by comparing the responses of standards of derivatised alcohols in mobile phase directly injected onto the HPLC column with those of extracted alcohols from plasma standards (here, alcohols were also derivatised after extraction). Extraction recoveries for methanol, ethanol and 1-propanol at six different concentrations (5.0, 10.0, 20.0, 50.0, 80.0, and 100.0 ng/ml) were evaluated. It was found that extraction recoveries were $91.5\pm4.7\%$ for methanol, $95.4\pm3.8\%$ for ethanol and $101\pm3.4\%$ for 1-propanol. The calculated limits of detection (at a signal-to-noise ratio of 3:1) for per injection were 0.1 pg for methanol, 0.2 pg for ethanol and 0.4 pg for 1-propanol, respectively. Even lower detection limits should be possible, but the practically obtained values are limited by the purity of the employed reagents during the derivatisation.

3.7. Separation and determination of alcohols

Fluorescence measurement yields excitation and emission maximum for 1-octanol-CRA derivative was 335 nm and 360 nm, respectively. When other derivatives were tested, similar maximum wavelengths were obtained. The detector was therefore operated at these excitation and emission wavelengths. For the separation of derivatised alcohols, several mobile phase compositions were tested. They included methanol, ethanol and acetonitrile in aqueous mixtures. For the simultaneous separation of alcoholic derivatives with 1–15 carbon atoms, a C_{18} column eluted with a acetonitrile gradient in water containing 0.1% acetic acid and 0.1% triethylamine to give the best separation with the shortest retention times and the sharpest peaks. Under these conditions, all 15 alcoholic derivatives were separated within 20 min (Fig. 3).

Fig. 4 demonstrates the application of this method for the determination of alcoholic contaminants present in a commercial potassium salt of alkyl phosphate ester. Here cetanol-CRA was used as an internal standard. The determined alcoholic contents of C_{11}, C_{12}, C_{13}, C_{14}, C_{15} were 7.4, 123.4, 18.7, 26.8 and 14.3 μg/g, respectively.

The detector responses for secondary alcohols 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. [33] and Haj-Yehia and Benet [27] by the use of 2-(4-carbox-

Table 4
Experimental and computed log k' values for alcohols, derivatised with CRA, on Spherisorb C_{18} (5 μm) column with ACN–water and MeOH–water (X% percentage of volume)

Alcohol n_c	X%	Log k' (ACN–water)		|Relative error| (%)	Log k' (MeOH–water)		|Relative error| (%)
		Exp.	Comp.		Exp.	Comp.	
1	45	0.34	0.31	8.8			
	50	0.23	0.21	8.7	0.74	0.73	1.4
	55	0.12	0.10	8.3	0.57	0.57	0.0
	60	0.03	0.02	33	0.43	0.42	2.3
	65				0.29	0.27	6.9
	70				0.12	0.11	8.3
2	45	0.51	0.51	0.0			
	50	0.39	0.39	0.0	0.90	0.91	1.1
	55	0.26	0.27	3.8	0.72	0.75	4.2
	60	0.16	0.16	0.0	0.57	0.59	3.5
	65	0.03	0.03	0.0	0.42	0.43	2.4
	70				0.27	0.27	0.0
3	45	0.70	0.71	1.4			
	50	0.57	0.58	1.8	1.08	1.10	1.9
	55	0.42	0.42	0.0	0.90	0.93	3.3
	60	0.30	0.32	6.7	0.72	0.77	6.9
	65	0.17	0.17	0.0	0.58	0.60	3.4
	70				0.40	0.43	7.5
4	45	0.90	0.87	3.3			
	50	0.76	0.77	1.3	1.35	1.30	3.7
	55	0.61	0.62	1.6	1.17	1.12	4.3
	60	0.45	0.48	6.7	0.98	0.95	3.1
	65	0.30	0.32	6.7	0.80	0.77	3.8
	70	0.16	0.17	6.3	0.62	0.59	4.8
	80				0.26	0.24	7.7
5	45	1.13	1.13	0.0			
	50	0.96	0.96	0.0	1.50	1.49	0.6
	55	0.79	0.79	0.0	1.31	1.31	0.0
	60	0.63	0.64	1.6	1.12	1.12	0.0
	65	0.47	0.47	0.0	0.93	0.93	0.0
	70	0.31	0.31	0.0	0.75	0.75	0.0
	80				0.39	0.39	0.0
6	45	1.33	1.33	0.0			
	50	1.20	1.20	0.0			
	55	1.00	1.01	1.0	1.45	1.48	2.0
	60	0.80	0.84	5.0	1.26	1.29	2.4
	65	0.60	0.65	8.3	1.07	1.10	2.8
	70	0.45	0.46	2.2	0.88	0.91	3.4
	80				0.50	0.53	6.0
7	50	1.39	1.34	3.6			
	55	1.18	1.15	2.5			
	60	0.96	0.97	1.0	1.49	1.47	1.3
	65	0.76	0.76	0.0	1.28	1.27	0.8
	70	0.57	0.57	0.0	1.07	1.07	0.0
	80				0.71	0.68	4.2

Table 5
Precision and accuracy for the analysis of 1-dodecanol ($n=6$)

	Concentration (theoretical) (ng/ml)	Concentration (found) (ng/ml)	RSD (%)	Recovery (%)
Intra-day	10	9.58	2.8	95.8
	40	38.4	3.3	96.0
	100	94.8	4.1	94.8
Inter-day	10	9.62	3.7	96.2
	40	38.8	3.9	97.0
	100	95.6	4.1	95.6

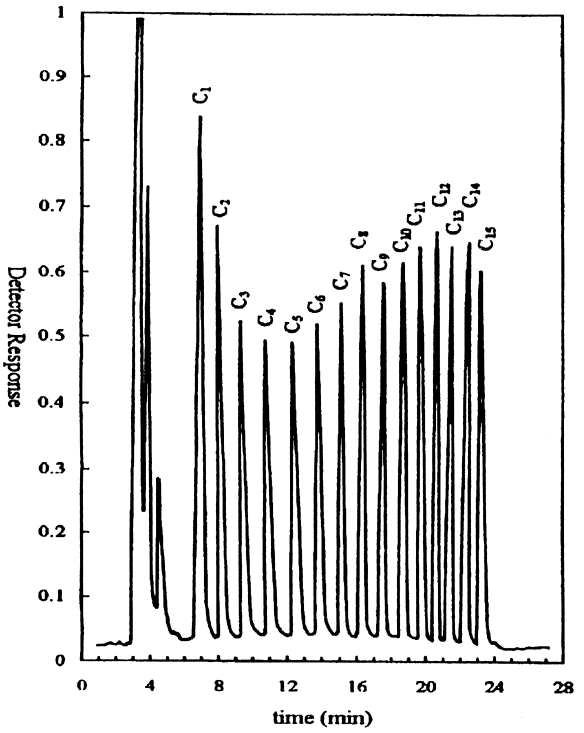

Fig. 3. Chromatogram of CRA-esters of C_1–C_{15}. A mixture consisting of 10 ng each of the alcohols was derivatised with CRA as described in the text. Detection with fluorescence (excitation 335 nm, emission 360 nm). Chromatographic conditions: column, 200×4.6 mm I.D. Spherisorb 5 μm; eluent: (A) 0.1% acetic acid+0.1% triethylamine–acetonitrile (65:35, v/v); (B) acetonitrile–water (95:5, v/v); flow-rate=1.0 ml/min; column temperature 30°C. Gradient conditions: initial=95% A; 20 min=20% A; 25 min=5% A; 30–40 min=100% B. C_1=Methanol, C_2=ethanol, C_3=1-propanol, C_4=*n*-butanol, C_5=1-pentanol, C_6=1-hexanol, C_7=*n*-heptanol, C_8=1-octanol, C_9=nonanol, C_{10}=decanol, C_{11}=undecanol, C_{12}=dodecanol, C_{13}=tridecanol, C_{14}=tetradecanol, C_{15}=pentadecanol.

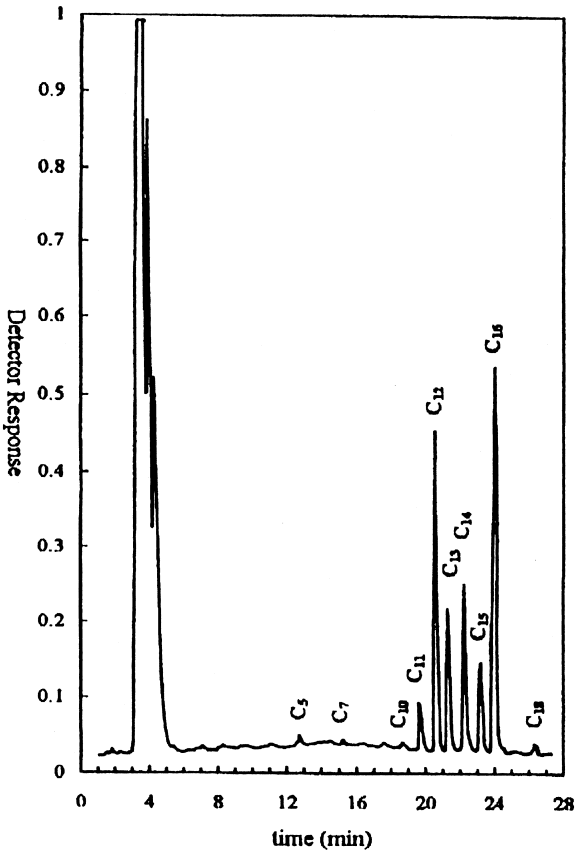

Fig. 4. Chromatogram for the determination of alcoholic contaminants from a commercial potassium salt of alkyl phosphate ester. Alcoholic contaminants were extracted as described in the text; cetanol-CRA (C_{16}) was used as an internal standard; peaks as in Fig. 3.

yphenyl)-5,6-dimethylbenzimidazole and 2-(4-carboxyphenyl)-6-methoxybenzofuran as the derivatisation reagents, respectively.

The determination of volatile alcohols from human plasma was carried out by two ways: (1) plasma was centrifuged and directly derivatised without further treatment; (2) the volatile alcohols from plasma samples were first extracted by chloroform and then derivatised, these results are showed in Table 6, respectively. As can be seen from Table 6, the contents of methanol and ethanol from the extracted plasma samples are slightly lower than that from the real plasma samples. It is probably due, in part, to the fact that methanol and ethanol in real plasma samples are of high water solvation with difficulty extracted by chloroform. In addition, the derivatisation with chloroform as co-solvent results in low yields (the chromatogram for the separation of volatile alcohols extracted from plasma is not showed). The real sample chromatogram (sample No. 3 not extracted with chloroform) is shown in Fig. 5 (sample Nos. 1 and 2 not shown).

3.8. Effect of other substances

EDC·HCl was a powerful coupling agent for the preparation of acidamide compounds in organic synthesis under mild conditions, where anhydrous solvents was not required [34]. In this study, the interference from organic amines in general, needs to be considered. It was found that many amines including some simple secondary amines such as

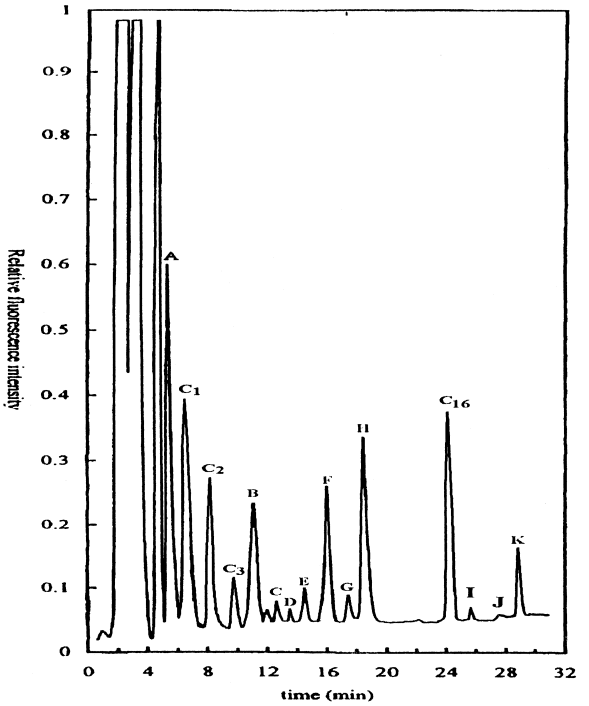

Fig. 5. Chromatogram for the determination of volatile alcohols in real human plasma sample by the reaction of cabazole-9-acetic acid with alcohols using DEC·HCl as derivatisation agent in the presence of DMAP. Elution and chromatographic conditions as in Fig. 3. C_1 = Methanol, C_2 = ethanol, C_3 = 1-propanol, C_{16} (cetanol) was used as an internal standard; other components A~K are not identified.

Table 6
The contents of volatile alcohols in spiked plasma (contents calculated on an average, $n=5$)

Sample No.[a]	Alcohol	Real plasma sample (μmol/ml) (determined)	Added (μmol/ml)	Found (μmol/ml)	RSD (%) ($n=5$)	Recovery (%)
1	Methanol	21.8[b] (26.7)[c]	20.0	40.4	3.7	96.6
	Ethanol	14.4[b] (18.9)[c]	15	30.2	3.2	103
	1-Propanol	6.1[b] (10.6)[c]	5	12.1	3.7	109
2	Methanol	17.4[b] (24.5)[c]	20.0	36.8	4.0	98.4
	Ethanol	15.2[b] (22.7)[c]	15	31.8	3.7	105
	1-Propanol	1.3[b] (8.4)[c]	5	7.8	2.8	124
3	Methanol	20.8[b] (27.7)[c]	20.0	39.7	4.0	97
	Ethanol	6.7[b] (14.5)[c]	15	23.4	3.6	108
	1-Propanol	10.2[b] (20.6)[c]	10	22.8	3.4	113

[a] Plasma samples were friendly obtained from Qufu Traditional Chinese Medical Hospital (China).
[b] Plasma samples were extracted by chloroform.
[c] Plasma samples were centrifuged without treatment and directly derivatised.

dimethylamine and diethylamine reacted with CRA under the derivatisation conditions proposed. However, the reactivities of most secondary amines were at least one order of magnitude less than that of primary amines possibly due to secondary amines imposing two alkyl groups led to more spatially demanding configurations. Generally, the 10- to 50-fold excess of secondary amines did not interfere with the derivatisation of most alcohols (straight chain alcohols). The interference from primary amines could be easily eliminated by means of the extraction purification as described above for the extraction of alcohols from the potassium salt of alkyl phosphate ester.

4. Conclusions

CRA reacts with alcohols in the presence of coupling agent and catalyst to form stable CRA-esters, which are not only sensitive for fluorimetric detection but also a very convenient procedure for the indirect determination of alcohols. Compared with currently available methods for the determination of alcohols, the proposed method offers a number of advantages: anhydrous reaction conditions are not required for the derivatisation procedure or storage of the reagents. For the most of methods mentioned in the text, in particular, acyl chlorides are neither satisfactory nor convenient because of their instabilities towards moisture. CMABS [25], previously reported in our study, is available the derivatisation reagent for the determination of alcohols, but derivatisation is only limited to C_1–C_9 probably due to a substituted 2-methyl group leading to a large steric hindrance. CRA molecule contains a nitrogen atom, n–π conjugation (nitrogen atom is an electron pair donor) in molecule is greatly augmented which significantly increases the detection sensitivity and reactivity for the derivatised alcohols. Comparing the structure of CRA with CMABS, it is crucial that 2-methyl group of CMABS molecule is thoroughly substituted by hydrogen atom and converted its molecule to CRA with little steric hindrance. The derivatisation of CRA with alcohols is facile, inexpensive, sensitive and reproducible. Complete derivatisation of further higher carbon alcohols (C_{17}–C_{20}), in fact, can easily be achieved under the HPLC derivatisation conditions proposed. The reaction of the reagent with amines is inevitable, but the interference from amines can be eliminated by the purification treatment as described in text. A possible disadvantage of the proposed method is that the reagent can only be used in the pre-column mode, and the levels are in the pg range. The trace-level application including the purity of the employed reagents would be reported elsewhere.

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