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Retention behaviour and fluorimetric detection of procaine hydrochloride using carboxymethyl-β-cyclodextrin as an additive in reversed-phase liquid chromatography

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Abstract

The retention behaviour of procaine hydrochloride on an Alltima octadecyl silica (C_{18}) column, with a mobile phase containing negatively charged carboxymethyl-β-cyclodextrin (CM-β-CD), influenced by a combination of hydrophobic and electrostatic interactions was systematically investigated. Various factors affecting the retention of procaine on the C_{18} column such as the concentration of CM-β-CD, pH and the methanol percentage in the mobile phase, were studied. An equation was applied to estimate the apparent binding constant of the CM-β-CD–procaine inclusion complex as an aid for understanding the retention mechanism. The first analytical application of CM-β-CD as a mobile phase additive for the determination of procaine was developed. The calibration curve was linear in the range 22–1360 ng ml^{-1} with an RSD of 2.1%. The detection limit based on 3σ was 1 ng ml^{-1} with fluorimetric detection at the excitation and emission wavelengths of 305 nm and 350 nm, respectively. The limit of quantitation based on 10σ was 22 ng ml^{-1}. The proposed method has been successfully applied to real sample analysis.

Keywords: Fluorimetric detection; Detection, LC; Procaine; Carboxymethyl-β-cyclodextrin

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides with the torus-shaped structure consisting of a relatively hydrophobic cavity and polar exterior. The basic property of CDs that allows them to affect numerous chemical separations is their ability to form selective inclusion complexes with a variety of sizes suitable for guest molecules of neutral or ionic nature [1,2]. These guest molecules can be partially or fully incorporated in the hydrophobic interior of the CDs, which forms the main reason for their wide applications in chemistry and in separation technology [3]. In addition, the exterior hydroxyl groups of CDs can be chemically modified to produce derivatised neutral or anionically or cationically charged CDs with the inclusion properties different from the parent ones. CDs may act as chiral resolving agents in the stationary phase, when chemically bonded to silica [4–8] or dissolved in the mobile phase as modifiers [9–11] or even in more complex separations [12]. Up to now native CDs such as α-, β-,

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γ-CDs [13–15] and their methylated [16,17] and hydroxypropylated derivatives [18,19] have been used successfully in many applications. Most applications focus on the analytical-scale separations. These CDs are usually not charged in a pH range 2–11. The complex-forming interaction is due to hydrophobic interactions between the analyte and the CD cavity and hydrogen bonding of the analyte to the hydroxyl groups or other introduced functionalities on the CD ring.

The use of charged CDs opens a new field of research and is especially suitable for high-performance liquid chromatography (HPLC) [20] or capillary electrophoresis (CE) [21,22] as mobile phase/buffer additives because of their additional potential electrostatic interactions with the analytes. Charged CDs exhibit a broad range of desirable properties such as: (1) optical inertia with little or no absorption in the UV region; (2) non-toxicity; and (3) increased solubility under the optimum pH. The most commonly used charged CDs are carboxymethylated and sulfobutylated β-CDs. Sulfobutyl ether, sulfopropyl ether and sulfoethyl ether CDs have recently been introduced and proved to be very useful for chiral separations [22–25]. Carboxymethyl and carboxyethyl-β-CDs have been used as mobile phase additives to achieve discrimination for enantiomers of drugs [20,26]. Numerous studies employing natural or neutral CD as mobile phase additives in HPLC have indicated that retention usually becomes shorter in the presence of the neutral CD, reflecting that the interaction between the solute and the stationary phase is weakened by complex formation [27,28]. Since the change of the retention value caused by the formation of the complex is closely related to the complex stability, some attempts have been made to estimate the binding or dissociation constant of the complex from the relationship between the retention value and the concentration of neutral CD in the mobile phase [29–32]. However, to date only a few reports have focused on the explanation of retention mechanisms involved under reversed-phase HPLC modified with charged CDs.

Procaine is used as a local anaesthetic in a wide variety of pharmaceutical preparations as an injection with antibiotic properties. Many analytical methods have been developed for the quantification of procaine hydrochloride [33–37]. The most recently reported method is based on permanganate-induced chemiluminescence detection [38]. However, the sensitivity and limit of detection are still not satisfactory. In our previous work [39], we have investigated the inclusion behaviour of charged carboxymethyl-β-cyclodextrin (CM-β-CD) with procaine by steady state enhanced fluorimetry. The strong inclusion property of CM-β-CD is considered to be due to the additional electrostatic interaction of a negatively charged CM-β-CD molecule and a cationic procaine molecule. However, in real life situations, sample possessing complicated matrices will generate serious interferences on the steady state fluorimetric measurements and subsequently hamper their practical applications. To overcome the matrix effect, it is preferred that the analyte is separated from its own sample matrix before actual measurements are performed. So far the most powerful and successful technique in tackling this problem mainly relies on chromatography and CE.

The aim of this work is to examine the retention behaviour of procaine hydrochloride in reversed-phase HPLC involving CM-β-CD inclusion complex formation in the mobile phase. Taking into account the enhanced fluorescence property of procaine hydrochloride in CM-β-CD medium, we attempt to develop an HPLC method to improve procaine determination in terms of sensitivity and limit of detection. The proposed HPLC method demonstrates successfully the determination of procaine in several pharmaceutical preparations. The application of CM-β-CD in HPLC also provides us with a better understanding of the retention mechanisms, i.e., the interactions between the mobile phase, the stationary phase, and the CM-β-CD–solute complex.

2. Experimental

2.1. Chemicals and reagents

Methanol (MeOH, HPLC grade) was obtained from Labscan Asia (Bangkok, Thailand). Procaine hydrochloride (>99%) was from Acros Organics (Geel, Belgium). CM-β-CD was prepared according to the method of Reuben et al. [40], in an alkaline solution with monochloroacetic acid. The resultant was purified by twice precipitating with MeOH and

drying at 90°C under vacuum. The product was further purified with free chloride less than 0.1% by an ion-exchange column. The degree of substitution (4.9±0.3 carboxylates per molecule of CM-β-CD) was determined by titrating the carboxylate groups with perchloric acid in acetic acid. The results of elemental analysis (C 38.4%, H 5.4%) and flame photometry (Na 8.0%) were in agreement with that of the degree of substitution. The product has been successfully used in steady state fluorimetry [39]. Pharmaceutical injection solution samples were purchased from Shanghai Sunrise Pharmaceutical (Shanghai, China). All other reagents were of analytical-reagent grade and mobile phase solutions were prepared in deionised water.

2.2. Instrumentation

The liquid chromatograph used was a HP1050 series consisting of a pumping system, a vacuum degasser, an HP1046A programmable fluorescence detector (Hewlett-Packard, Wilmington, DE, USA) and a 7125 Rheodyne injector with a 20-μl sample loop. The chromatographic column was a 250×4.6 mm I.D. stainless steel tubing packed with 10 μm octadecyl bonded silica (Alltima C_{18}; Alltech, Deerfield, IL, USA). The steady state fluorescence spectra were measured on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan).

2.3. Chromatographic procedures

The mobile phase, i.e., a MeOH–water solvent mixture containing CM-β-CD in various concentrations, was prepared by dissolving known amounts of CM-β-CD in deionised water and then mixing it with MeOH. The pH of the aqueous CM-β-CD solution was adjusted by either 0.6 *M* HCl or 0.1 *M* NaOH and the pH measurements were taken on an Orion (Chicago, IL, USA) combined pH glass electrode. The mobile phase thus obtained was filtered through a 0.45-μm pore size membrane and sonicated for approximately 15 min prior to use. All chromatograms were obtained at 20°C at a flow-rate of 0.8 ml min^{-1}. The excitation and emission wavelengths of fluorescence detection were taken at 305 nm and 350 nm, respectively. The column void volume was determined using deionised water.

3. Results and discussion

3.1. Determination of apparent binding constants of procaine hydrochloride with CM-β-CD

The HPLC method enables the acquisition of inclusion complex binding constant even though the analyte is not in a pure form. A knowledge of the binding constant for the CM-β-CD–procaine inclusion complex is of interest first to appreciate the extent of the inclusion of procaine into the cavity of CM-β-CD and also to understand those factors influencing the inclusion process. These factors play important roles in the development of the HPLC method in conjunction with CM-β-CD as an additive in mobile phase. The relationship between k' and $[\text{CM-β-CD}]_T$ for a CM-β-CD–procaine complex of 1:1 stoichiometry [14,16,27] can be described by the following equation:

$$\frac{1}{k'} = \frac{1}{\phi K_0} + \frac{K_f[\text{CM-β-CD}]_T}{\phi K_0} \quad (1)$$

where k', ϕ, K_0, K_f and $[\text{CM-β-CD}]_T$ are the retention factor of procaine hydrochloride, the phase ratio (the ratio of the volume of stationary phase to the volume of mobile phase in the column), the distribution constant of procaine hydrochloride in the stationary phase to the mobile phase, the apparent binding constant and the total concentration of CM-β-CD in the mobile phase, respectively.

Fig. 1 shows plots of $1/k'$ versus $[\text{CM-β-CD}]_T$ for procaine hydrochloride in an MeOH–water (3:7, v/v) mobile phase under various pH conditions. It has been reported that procaine hydrochloride can form a 1:1 stoichiometric inclusion complex with β-CD [41]. A good linear relationship is observed for most calibration curves which probably suggests that the CM-β-CD–procaine complexation reaction is predominantly of a 1:1 stoichiometry. Table 1 displays the calculated apparent binding constants of CM-β-CD–procaine complexes at different pH values. At pH 5.3, the binding constant is largest which indicates that there is the strongest inclusion interaction between CM-β-CD and procaine molecules.

Fig. 2 displays the corrected fluorescence excitation and emission spectra of 10 μ*M* procaine in MeOH–water (3:7, v/v, pH 6.0) obtained from a

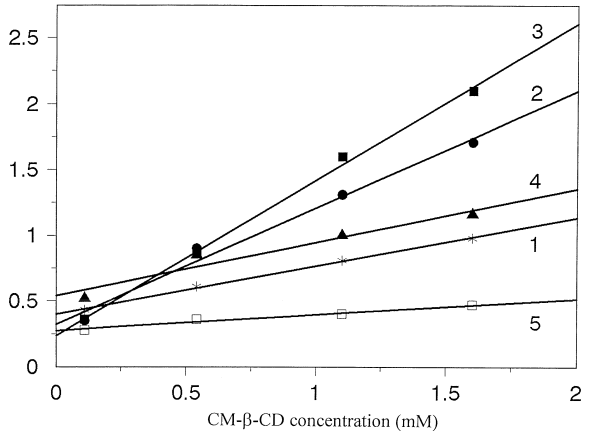

Fig. 1. Plot of $1/k'$ versus concentration of CM-β-CD using 1 μ*M* procaine hydrochloride and a mobile phase of MeOH–water (3:7, v/v) containing CM-β-CD at various pH values. (1) 3.3; (2) 4.4; (3) 5.3; (4) 6.0 and (5) 6.5.

spectrofluorimeter. The fluorescence intensity of procaine increases with the concentration of CM-β-CD in the MeOH–water (3:7, v/v) solvent mixture. It is obvious that the formation of an inclusion complex between CM-β-CD and procaine can increase the fluorescence intensity. Similar fluorescence enhancement effect of procaine in the presence of CM-β-CD was observed at other pH values. The K_f values can be determined from the steady state fluorescence measurement using a modified equation from Catena and Bright [42]:

$$\frac{1}{F-F_0}=\frac{1}{q[\mathrm{A}]_\mathrm{T}}+\frac{1}{q[\mathrm{A}]_\mathrm{T}K_\mathrm{f}[\text{CM-β-CD}]_\mathrm{T}} \qquad (2)$$

where F and F_0 are the fluorescence intensity in the presence and absence of CM-β-CD in the solution, respectively. $[\mathrm{A}]_\mathrm{T}$ is the total procaine concentration and q is a constant combining all the quantum yields of free procaine and CM-β-CD–procaine complex and the instrumental constant. From Eq. (2), a plot of $1/(F-F_0)$ versus $1/[\text{CM-β-CD}]_\mathrm{T}$ should be linear and have a slope of $1/q[\mathrm{A}]_\mathrm{T}K_\mathrm{f}$ and a y-intercept of $1/q[\mathrm{A}]_\mathrm{T}$. The apparent binding constant K_f for a CM-β-CD–A complex of 1:1 stoichiometry can be derived from the intercept to the slope ratio of the regression line. Fig. 3 shows plots of $1/(F-F_0)$ versus $1/[\text{CM-β-CD}]_\mathrm{T}$ for procaine in a solvent mixture of MeOH–water (3:7, v/v) under various pH conditions. A good linear relationship is observed for most calibration curves which suggests that the CM-β-CD–procaine complexation reaction is predominantly of a 1:1 stoichiometry. Table 1 displays the calculated apparent binding constants of CM-β-CD–procaine complexes at different pH values using the steady state fluorescence method. At pH 6.0–6.5, the binding constant is largest which indicates that there is the strongest inclusion interaction between

Table 1
The apparent binding constants K_f of CM-β-CD–procaine inclusion complex in a solvent mixture of MeOH–water (3:7, v/v) at various pH conditions determined by HPLC and steady state fluorescence methods

pH	Apparent binding constant, K_f (M^{-1})	
	HPLC method	Steady state fluorescence method
3.3	964±40	332±27
4.4	3529±660	418±17
5.3	5002±961	753±47
6.0	904±121	1996±29
6.5	478±20	2022±137
7.5	–	915±58

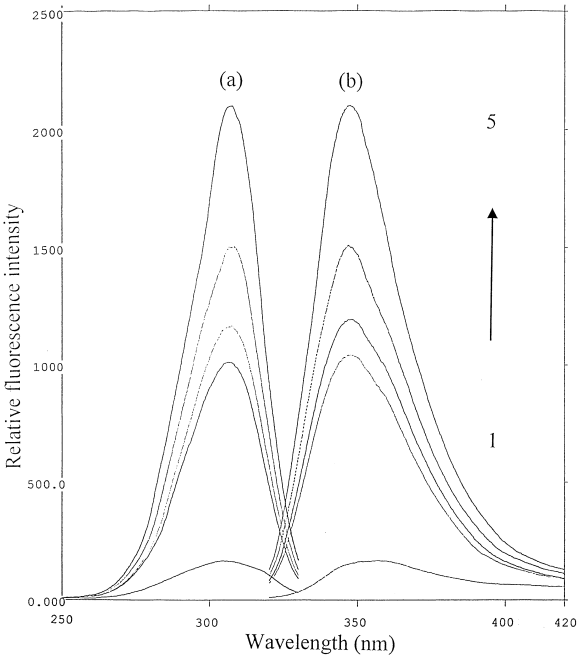

Fig. 2. Corrected fluorescence (a) excitation and (b) emission spectra of 10 μ*M* procaine hydrochloride in MeOH–water (3:7, v/v, pH 6.0) containing various concentrations of CM-β-CD. (1) 0.0; (2) 0.30; (3) 0.50; (4) 1.0 and (5) 3.0 m*M* CM-β-CD.

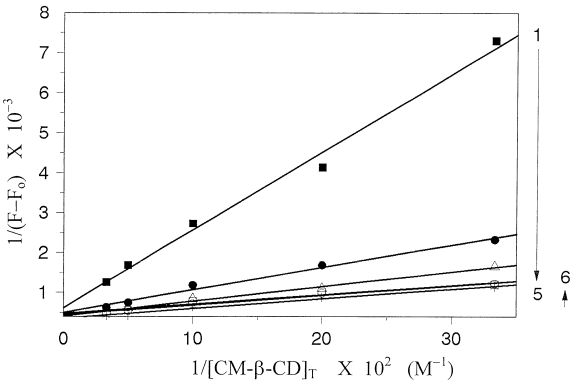

Fig. 3. Plot of $1/(F-F_0)$ versus $1/[\text{CM-β-CD}]_T$ using 10 μ*M* procaine hydrochloride in a solvent mixture of MeOH–water (3:7, v/v) containing CM-β-CD at various pH values. (1) 3.3; (2) 4.4; (3) 5.3; (4) 6.0; (5) 6.5 and (6) 7.5.

CM-β-CD and procaine molecules at this pH range under steady state fluorescence measurement. In general, the binding constant increases first with pH and then decreases at higher pH values for both HPLC and steady state fluorescence methods. The effect of pH on the formation of CM-β-CD–procaine complex will be discussed in the subsequent section. The highest binding constants for HPLC and steady state fluorescence methods occur at pH 5.3 and 6.0–6.5, respectively. It is interesting to note that the binding constants obtained from the HPLC method are generally higher than those from the steady state fluorescence method. This distinction is mainly due to the difference in experimental conditions used to obtain the binding constants.

3.2. Effect of pH on the retention and fluorescence

The effect of pH on the retention factor and fluorescence peak area of the CM-β-CD–procaine complex was studied since CM-β-CD and procaine can exist in different forms under various pH values. The pK_a values for CM-β-CD and procaine are 5–7 and 8.95, respectively [20,43]. The retention factor of procaine hydrochloride in an MeOH–water containing 2.3 m*M* CM-β-CD (3:7, v/v) mobile phase has a minimum in the pH range 4.4–5.3 whereas the fluorescence peak area reaches its highest value at pH 5.3. Actually, this is a complicated process involving the interactions of different molecule species. At pH<4, most carboxylic groups of CM-β-CD are protonated. At pH>4, deprotonation of the carboxylic groups leads to the formation of negatively charged CM-β-CD in the mobile phase. For procaine, it exhibits as positively charged species under the experimental pH range 3.3–6.5. The strong inclusion interaction between CM-β-CD and procaine at pH 5.3 to some extent results from the combination of hydrophobic and the additional electrostatic interaction. The electrostatic attraction arises from the negatively charged carboxymethyl group of CM-β-CD and the positively charged quaternary ammonium cation of procaine. In contrast to the secondary hydroxyl groups locked into the position on the native β-CD, the carboxymethyl group of the CM-β-CD is free to rotate. This flexibility may allow for a closer approach between the quaternary ammonium moiety of the procaine leading to stronger or more stereospecific interactions than are possible with a native β-CD. The hydrophobic interaction originates from the hydrophobic cavity of CM-β-CD and the *p*-aminobenzoate moiety of procaine. However, at higher pH values (>6), more carboxymethyl groups on the torus rim of the CM-β-CD are deprotonated to negatively charged carboxymethyl groups which will have an adverse effect on preventing the hydrophobic *p*-aminobenzoate moiety of a procaine molecule in entering into the CM-β-CD cavity [44]. As a result, the binding constant of CM-β-CD–procaine complex decreases at higher pH values. The relative fluorescence peak area increases in the pH range 3.3–5.3 and decreases at pH>5.3. It indicates that the formation of CM-β-CD–procaine complex can increase the fluorescence of procaine, which is extremely useful for improving the sensitivity and limit of detection of procaine determination by HPLC–fluorescence detection.

3.3. Effect of CM-β-CD as a mobile phase additive on retention and fluorescence intensity

The hydrophobic interaction, i.e., dispersion forces operating between the bonded alkyl moiety of the stationary phase and the non-polar part of the sample molecule, plays a crucial role in determining the retention value of the analyte in reversed-phase liquid chromatography. It is affected by various

factors such as the chain length and the amount of bonded alkyl moieties in the stationary phase, as well as the type and the content of the organic solvent and the additives in the mobile phase. When CM-β-CD was added to the mobile phase (MeOH–water, 3:7, v/v), the changes in the retention value and in the fluorescence peak area were due to the formation of the inclusion complex. The effect of the additive in the mobile phase on the retention and fluorescence peak area of procaine hydrochloride on a hydrophobic C_{18} column is shown in Fig. 4. The retention factor, k', of procaine hydrochloride considerably decreases and the fluorescence peak area increases upon the increase in CM-β-CD concentration. These results indicate that: (1) the decrease in k' value caused by the addition of CM-β-CD in the mobile phase is based on the formation of an inclusion complex, thus weakening the hydrophobic interaction between solutes and the stationary phase, and (2) the increase in fluorescence intensity is attributed to the protective micro-environment of the CM-β-CD cavity from quenching of procaine hydrochloride in the bulk MeOH–water solution. The increase in fluorescence peak area started levelling off at 2.3 m*M* CM-β-CD. Thus, this was the optimum CM-β-CD concentration chosen for most studies.

3.4. Effect of MeOH in the mobile phase on retention and fluorescence

The inclusion process for CM-β-CD in the liquid phase is easier to study in an aqueous solution than in an aqueous–organic solvent mixture. However, the use of an aqueous–organic solvent mixture as a mobile phase is essential for most HPLC applications. Firstly, in our preliminary experiments, a 100% aqueous CM-β-CD mobile phase resulted in a very long retention time (>20 min) for procaine on a C_{18} column which is actually impractical for real sample analysis. On addition of MeOH to the mobile phase, the retention time could be shortened to less than 10 min. It is usual to employ an MeOH–water mixture mobile phase for the reversed-phase HPLC system to obtain reasonable retention times for solutes. Secondly, the interaction between CM-β-CD and MeOH is so weak that the complexation of procaine with CM-β-CD is barely retarded by MeOH [27]. Thirdly, the solubility of CM-β-CD in MeOH is the largest among other straight chain alcohols. The effect of MeOH on the retention factor for procaine is shown in Fig. 5. The k' value decreases with the increase in the percentage of MeOH in the mobile phase. This finding is consistent with most

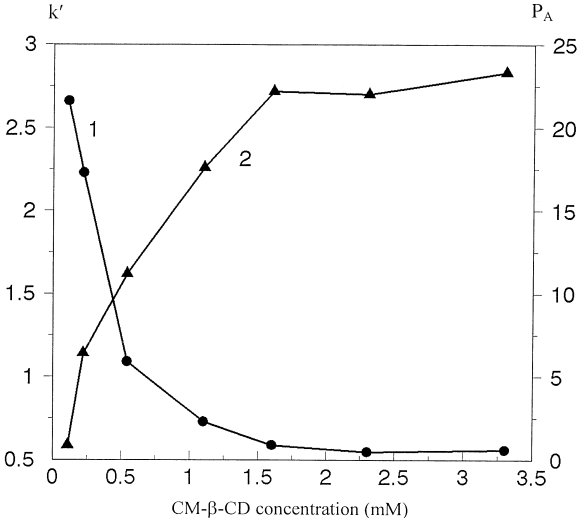

Fig. 4. Effect of CM-β-CD concentration on (1) the retention factor, k', and (2) the relative fluorescence peak area, P_A, using 1 μ*M* procaine hydrochloride and a mobile phase of MeOH–water (3:7, v/v) at pH 5.3.

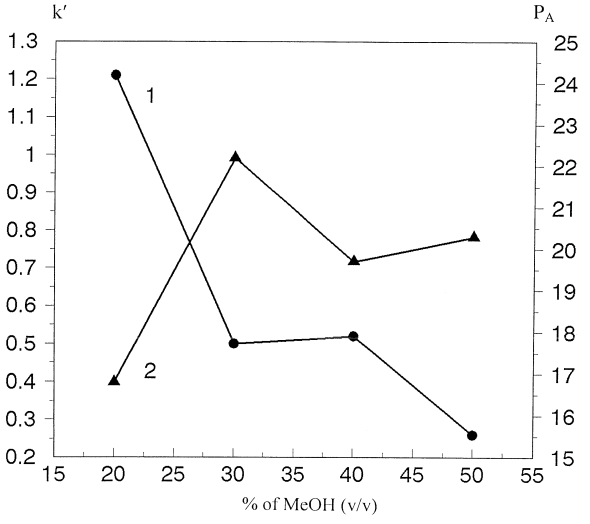

Fig. 5. Effect of MeOH percentage in the mobile phase on (1) the retention factor, k', and (2) the relative fluorescence peak area, P_A, using 1 μ*M* procaine hydrochloride and a mobile phase of MeOH–water containing 2.3 m*M* CM-β-CD at pH 5.3.

reversed-phase HPLC separations as the CM-β-CD–procaine inclusion complex is eluted faster at higher MeOH percentages in an MeOH–water mobile phase. There is also a slight enhancement in fluorescence peak area and these results are also consonant with the fluorescence intensity measurement using a spectrofluorimeter. However, too high MeOH content results in the precipitation of CM-β-CD in the mobile phase and also gives a very short retention time for procaine. The optimum MeOH content was then chosen at 30% in the mobile phase for sample analysis.

3.5. Analytical application

In the present work, procaine hydrochloride was chosen as a probe compound which can be hydrolysed in aqueous solution with the formation of *p*-aminobenzoic acid (PABA) [43]. Thus, it is worthwhile to consider the separation of procaine and PABA under the above-mentioned optimum chromatographic conditions. Fig. 6 displays the chromatographic separation of procaine and PABA at various pH values (3.3–6.5). The pH can affect the retention times for both procaine and PABA. It is interesting to note that the retention of PABA on a C_{18} column decreases with the increase in pH. Since the pK_a value of PABA is 4.9 [45], PABA exists mostly as an anion under higher pH values. With the increase in pH of the mobile phase, PABA molecules were deprotonated into anions resulting in less retention in a hydrophobic C_{18} column. The inclusion of PABA into CM-β-CD cavity became weaker due to the repulsive electrostatic force of PABA and CM-β-CD molecules [39]. The optimum pH value for sample analysis was chosen as 5.3 based on the combination of the baseline separation of procaine and PABA and analytical sensitivity. Consequently, the composition of the mobile phase was MeOH–water (30:70, v/v) mixture (pH 5.3) containing 2.3 m*M* CM-β-CD and used at a flow-rate of 0.8 ml min^{-1}. A linear calibration curve for procaine was obtained in the 22–1360 ng ml^{-1} range. The regression equation is $P_A = 1336.8C + 15.25$ with a correlation coefficient of 0.999 and a relative standard deviation (RSD) of 2.1% where P_A and C are the fluorescence peak area and the concentration of procaine hydrochloride, respectively. The detection

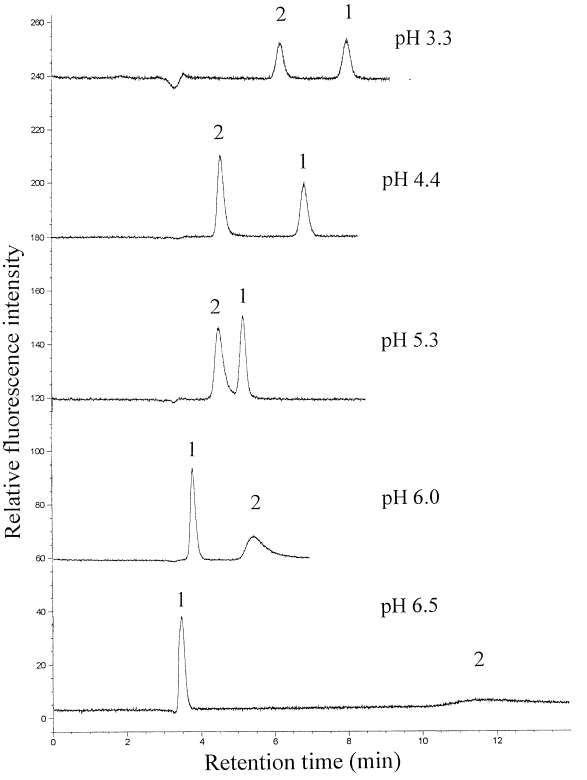

Fig. 6. Chromatographic separation of (1) 0.5 μ*M* *p*-aminobenzoic acid and (2) 0.5 μ*M* procaine using a mobile phase of MeOH–water (3:7, v/v) containing 2.3 m*M* CM-β-CD at various pH values.

limit based on 3σ (three times the standard deviation of blank) was 1 ng ml^{-1}. The limit of quantitation based on 10σ was 22 ng ml^{-1}. The proposed method was then applied to the determination of procaine hydrochloride in pharmaceutical injections. Since the procaine hydrochloride in the injections is in water-soluble form, the determination of procaine hydrochloride becomes very simple. The sample treatment is straightforward and involves only dilution with water to the appropriate concentration that falls within the calibration range. Procaine was well separated from the potential interferent PABA. No other interferents were found in the injections. The results for the determination of procaine hydrochloride in the pharmaceutical injections and the recovery tests are summarised in Table 2 and the results were satisfactory. It is obvious that the proposed HPLC method offers an excellent, accurate

Table 2
Analysis of procaine hydrochloride in pharmaceutical injections and the recovery test

Pharmaceutical injection	Declared value on the label (mg ml^{-1})	Content found[a] (mg ml^{-1})	Procaine added (μg ml^{-1})	Procaine found[a] (μg ml^{-1})	Recovery (%)
Sample 1	20	15.2±0.24	0.044	0.041±0.003	93
Sample 1	20	15.2±0.24	0.44	0.43±0.03	98
Sample 1	20	15.2±0.24	1.10	1.16±0.02	105
Sample 2	2.5	2.28±0.06	0.044	0.042±0.004	95
Sample 2	2.5	2.28±0.06	0.44	0.43±0.02	98
Sample 2	2.5	2.28±0.06	1.10	1.15±0.08	105

[a] The procaine content is an average of three determinations by the proposed HPLC method.

and precise method for the quantification of procaine in pharmaceutical injections with almost no effect of interferences from components in the sample solution.

4. Conclusion

Charged cyclodextrin is a very effective mobile phase additive in HPLC methods. The combination of hydrophobic and electrostatic interactions offers a wide range of possibilities for controlling retention. The retention behaviour of analytes not only depends on the concentration of charged CD, but also the pH and binding constants of inclusion complex. Liquid chromatography is also a technique that should complement spectroscopy in probing the mechanism of the inclusion phenomena of cyclodextrins. In addition, the significant spectral changes caused by inclusion complexation will imply potential analytical application. In summary, a HPLC method combined with enhanced fluorimetric detection for the separation and determination of procaine was developed. To the best of our knowledge, this is the most sensitive method for procaine determination reported to date. The proposed HPLC method is simple, rapid, sensitive and suitable for routine pharmaceutical analysis.

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