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DETERMINATION OF THIAMINE AND THIAMINE PHOSPHATES IN EXCITABLE TISSUES AS THIOCHROME DERIVATIVES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON OCTADECYL SILICA

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SUMMARY

The analysis of thiamine and thiamine phosphates by high-performance liquid chromatography owes its high sensitivity to the fluorescent derivatives or thiochromes obtained by chemical oxidation in alkaline medium. The possibility of performing precolumn oxidation with potassium ferricyanide instead of using the hazardous cyanogen bromide has been investigated. The derivatization step has been optimized with respect to the following parameters: concentration of alkali and oxidant, presence of methanol and stability of the thiochromes.

A gradient separation with 25 m*M* phosphate buffer (pH 8.4) and methanol as mobile phase components and an octadecyl silica column as stationary phase has been set up. The analytical run takes 14 min with the following elution order: thiochrome triphosphate, thiochrome pyrophosphate, thiochrome monophosphate and thiochrome. The minimum detectable amount is 0.05 pmol.

The method was found suitable for the determination of thiamine compounds in excitable tissues such as nerves and electric organs as well as in proteins extracted from membranes of these organs. It may be useful to study the role of thiamine in the electrical activity of these tissues at the molecular level.

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INTRODUCTION

Thiamine is present in animal tissues in different forms: thiamine (T), thiamine monophosphate (TMP), thiamine pyrophosphate or cocarboxylase (TPP) and thiamine triphosphate (TTP). Among these forms, TPP has a well known function as cofactor during enzymatic decarboxylation. However, considerable evidence has recently been accumulated in favour of a second function of thiamine compounds specific to the nervous system [1]. In addition to electrophysiological experiments more precise biochemical results suggest that the phosphorylated esters of thiamine could play a role in the electrical activity of excitable tissues, i.e. at the level of the voltage-dependent sodium-channel activity [2—4]. As this ion-gating mechanism has been described in terms of a phosphorylation—dephosphorylation cycle of a protein, TTP or TPP could be endogenous sources of phosphorus in this model [5—8]. To study this possibility we needed a rapid and sensitive technique to separate and quantify the thiamines at different stages of the sodium-channel preparation [9—11].

High-performance liquid chromatography (HPLC) seems to be the method of choice since its sensitivity reaches the subpicomole level using fluorimetric detection. Thiamines are converted into fluorescent derivatives or thiochromes by alkaline oxidation: Thc, ThcMP, ThcPP and ThcTP. In most of the recently published works [12—14], this derivatization is performed after the chromatographic separation, using $K_3Fe(CN)_6$ as oxidative reagent, which requires an additional experimental device: pump, mixing coil and incubator. In contrast, cyanogen bromide is used as oxidant in precolumn derivatization [15—17]. This chemical is very poisonous and its alkaline solutions are not stable. For these reasons, we have developed a simple method using $K_3Fe(CN)_6$ in precolumn derivatization. The separation is carried out with octadecyl silica as solid phase and mixtures of phosphate buffer and methanol as mobile phase. The technique is applied to the analysis of excitable tissue extracts.

MATERIAL AND METHODS

Instrumentation

The apparatus was an Altex (Berkeley, CA, U.S.A.) Model 334-50 programmable gradient liquid chromatograph consisting of two single-piston pumps (Model 110 A), a system controller (Model 421), a high-pressure solvent mixer and a sample injection valve (Model 210) with a 20-μl loop. Pump A was used to deliver the aqueous buffer and pump B the methanol.

The columns were also obtained from Altex (Ultrasphere-ODS, 5 μm, 150 or 250 × 4.6 mm). They were protected by precolumns (4.2 × 3.2 mm) dry-packed with Vydac-201RP (30—44 μm) from Macherey-Nagel (Düren, F.R.G.). At a flow-rate of 1 ml/min the resulting pressure varied from 7 to 17 MPa during the gradient elution.

Two detectors were connected in series. The Gilson Spectra/Glo filter fluorometer (Middelton, U.S.A.) was used with standard filters (excitation 390 nm, emission 475 nm) and cell (15 μl). The absorbance detector was an Altex Model 165 multichannel rapid scanning ultraviolet—visible apparatus.

Electrical signals were transfered to a double-pen recorder (10 mV) and/or a C-R1A Chromatopac Shimadzu integrator (Kyoto, Japan).

Reagents and solvents

T, TMP and TPP were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. TTP was prepared according to the method of Penttinen [18]. Aqueous solutions were prepared with water delivered by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.) and filtered on 0.45-μm ultrafilters. Organic solvents and other chemicals were of pro analysi grade (Merck, Darmstadt, F.R.G.).

Derivatization procedure

Standard mixtures of thiamines were dissolved in water. Aliquots were distributed in small tubes and lyophilized. The dried powder was stored at −20°C and the solution was reconstituted when necessary with 80 μl of water or water—methanol (1:1, v/v).

Optimal oxidation was obtained by adding 50 μl of fresh alkaline ferricyanide to 80 μl of methanolic standards, followed by a brief mixing (5—10 sec).

The alkaline ferricyanide solution was prepared daily as follows: 100 mg of $K_3Fe(CN)_6$ were dissolved in 10 ml of water; 50 μl of this solution were added to 2.5 ml of 15% NaOH. The resulting oxidant solution was found to be stable for one day if stored in the dark.

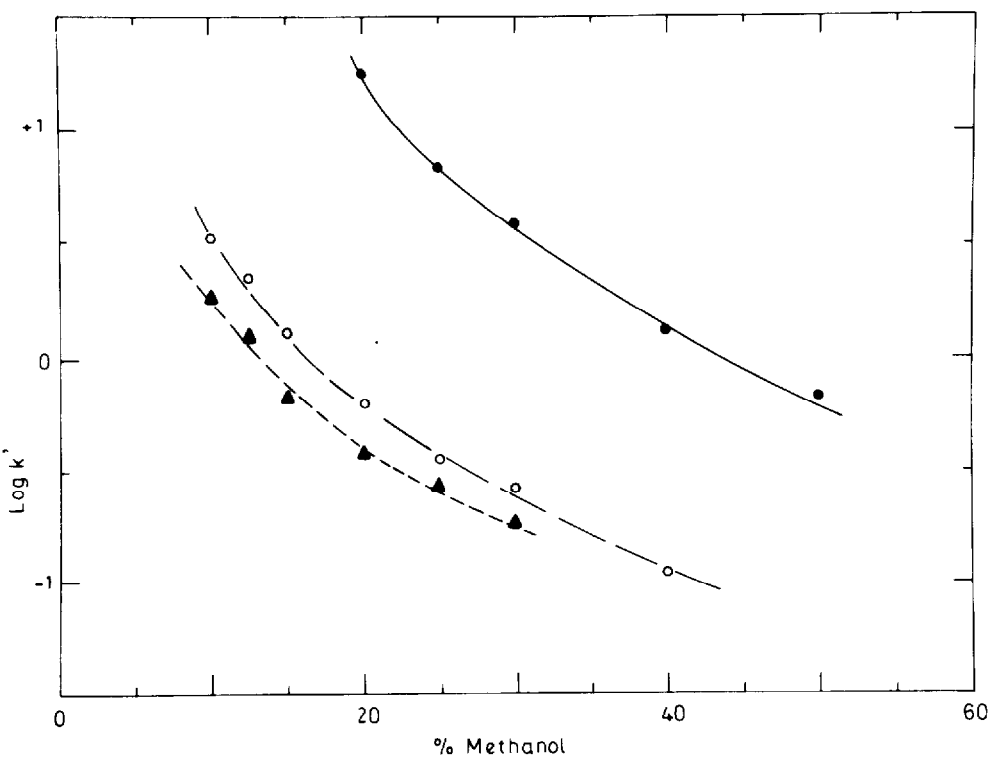

Fig. 1. Dependence of the logarithm of the capacity factor, k', on mobile-phase composition for thiochrome derivatives: (•), Thc; (○), ThcMP; (▲), ThcPP. Mobile phase: 25 mM potassium phosphate buffer (pH 8.4)—methanol. Stationary phase: Ultrasphere-ODS, 250 × 4.6 mm, 5 μm. Flow-rate: 1 ml/min.

Sample preparation

The procedure described by Ishii et al. [19] was followed except for the following points. Samples of 100 mg of tissue were homogenized in 3 vols. of 5% trichloroacetic acid at 4°C in a motor-driven glass homogenizer. After centrifugation (1 h, 5000 *g*, 4°C) the supernatant was extracted for 1 h with 3 vols. of water-saturated diethyl ether. Aliquots of the aqueous phase were oxidized as described above and injected 1 min after the addition of the $K_3Fe(CN)_6$—NaOH mixture.

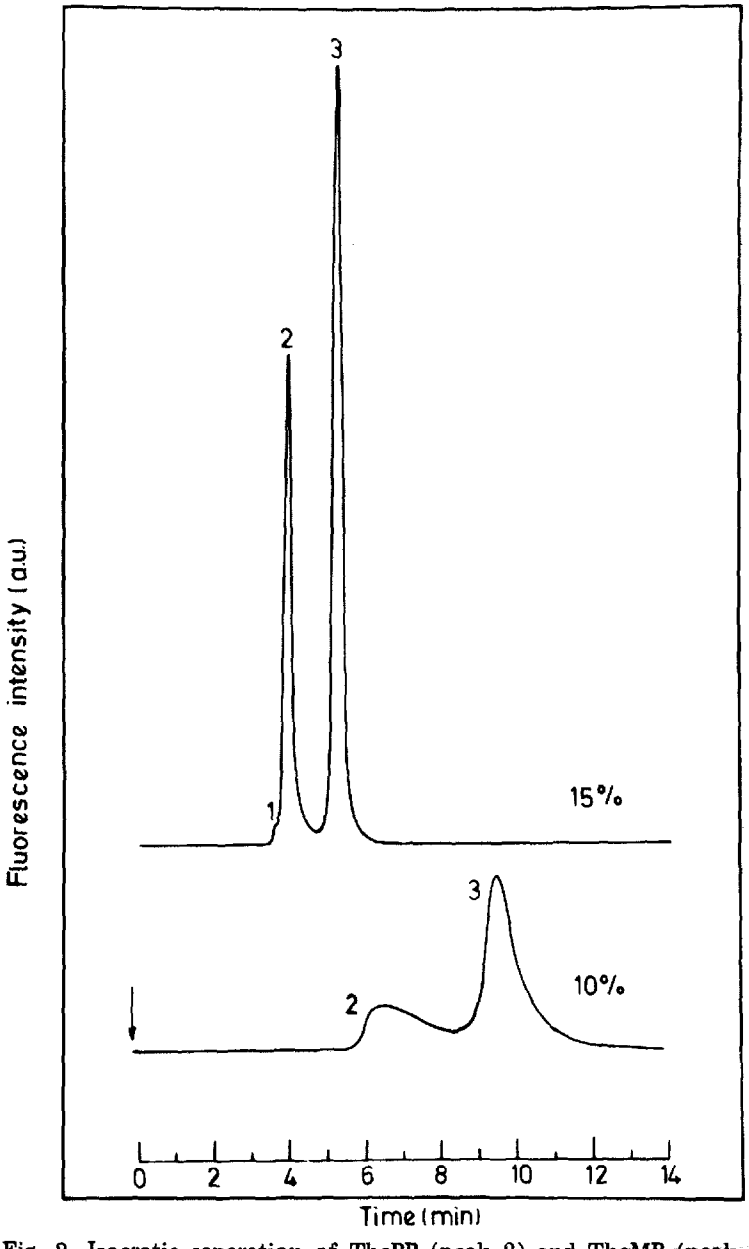

Fig. 2. Isocratic separation of ThcPP (peak 2) and ThcMP (peak 3) in 15% (upper part) followed by 10% methanol (lower part).

RESULTS AND DISCUSSION

Regulation of retention

Sanemori et al. [16] have shown that ThcTP, ThcPP and ThcMP can be separated isocratically in 20 min in the reversed-phase mode using 25 m*M* phosphate buffer (pH 8.4) and 2.5% N,N-dimethylformamide (DMF) as mobile phase. Thc could be subsequently eluted if the DMF content was increased to 50%. Our aim was to develop a method for the simultaneous determination of the four thiochromes by gradient elution using methanol as organic modifier instead of DMF.

The capacity factors were determined for the three major thiochromes at different methanol concentrations (Fig. 1). The higher retention of Thc compared to ThcMP and ThcPP clearly justifies the use of a gradient to shorten the analysis time. This experiment was carried out from a high methanol percentage (50%) to lower values. As the mobile-phase composition was changed from 15% to 10% methanol, a particular phenomenon was observed, both with phosphate (pH 8.4) or borate (pH 9.0) buffer (Fig. 2): considerably broader and deformed peaks were obtained without any modification of their areas. This effect could be suppressed either by repeated injections of the oxidized sample at short intervals (e.g. 1 min) or by a preinjection of the alkaline oxidant or of 15% NaOH alone.

Gradient set-up

The following gradient was programmed: after 1 min at 10%, the methanol concentration was raised to 100% in 3 min; 6 min after the injection the initial conditions were restored (Fig. 3B). Under these conditions a good resolution of the esters was obtained and thiamine eluted 1.5 min later. However, due to the very short re-equilibration time the methanol concentration is probably somewhat higher than 10% during the first step of the gradient. Obviously a first-gradient cycle without injection and a constant time interval between the injections (14 min) are required to obtain good reproducibility of the retention: $k' = 1.4$, 1.6, 2.0 and 3.8 for ThcTP, ThcPP, ThcMP and Thc, respectively. In the chromatograms presented in Fig. 3A, the small peak at 3.95 min (peak 1) was attributed to ThcTP as contaminant of the other products. This was confirmed later (Fig. 3B) when TTP was synthesized according to the procedure of Penttinen [18]. A frontal peak is observed by monitoring the absorbance at 254 nm (Fig. 3A) which is due to excess of the oxidant, $K_3Fe(CN)_6$. The minor peak between ThcMP (peak 3) and Thc (peak 4) is a fluorescent impurity present in the mobile phase since this peak also appears when the gradient is run without injection.

Optimization of the precolumn derivatization procedure

The oxidization conditions were found to be extremely variable in the literature [12—17, 20]: the final concentration of $K_3Fe(CN)_6$ varies from 1×10^{-4} to 30×10^{-4} *M*, that of NaOH from 0.04 to 1.9 *M*. We found it important to study by chromatographic means the influence of the following factors: reagent concentration, presence of methanol in the reaction medium and reaction time. The stability of the oxidized thiamines was also investigated. Peak areas were

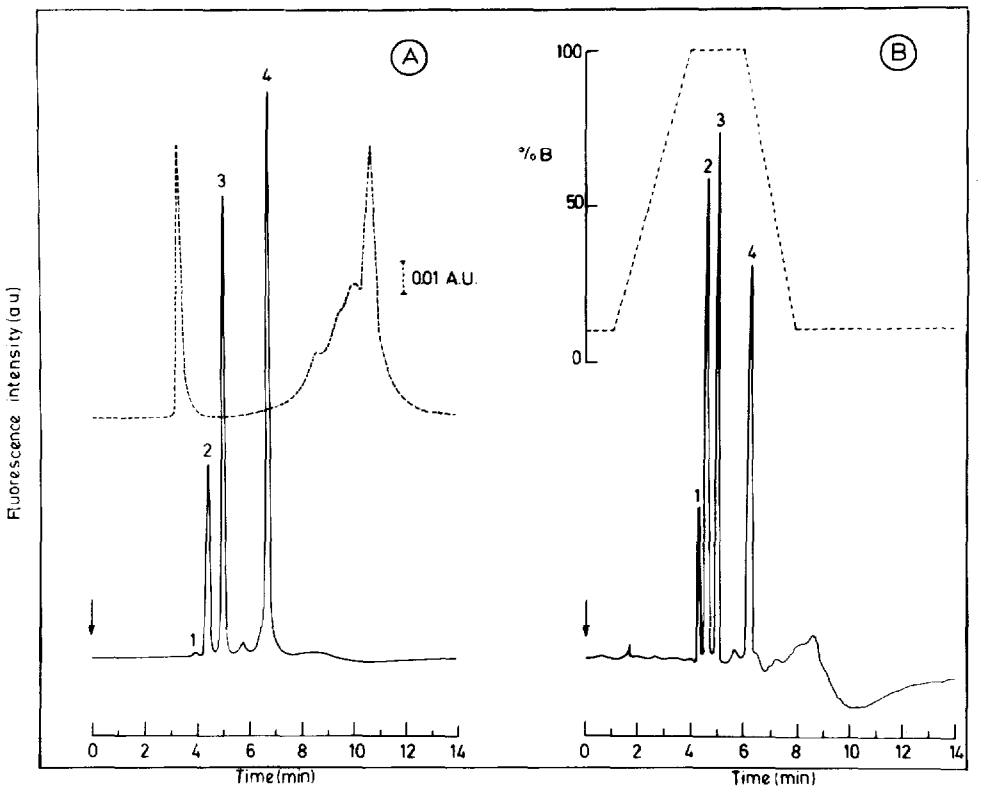

Fig. 3. Gradient separation of ThcTP (peak 1), ThcPP (peak 2), ThcMP (peak 3) and Thc (peak 4). Solid lines refer to the fluorescence signal and dotted lines to the UV trace at 254 nm (A) and to the profile of the programmed gradient (B).

measured from chromatograms corresponding approximately to 10 pmol of T and TMP and 6 pmol of TPP injected 1 min after the addition of the oxidizing mixture.

Fig. 4 illustrates the effect of the concentrations of the oxidizing reagents on the thiochrome production: $K_3Fe(CN)_6$ (Fig. 4A), NaOH (Fig. 4B) and combined chemicals (Fig. 4C). Constant values of peak areas are obtained at $K_3Fe(CN)_6$ concentrations higher than 0.3×10^{-4} *M* at a NaOH molarity of 1.44 (Fig. 4A). If the oxidant is maintained at a concentration of 1.54×10^{-4} *M* and NaOH varied, an important dependence upon NaOH concentration is observed (Fig. 4B): the peak areas increase to reach a maximum value above 1.5 *M*. This influence is also shown with more experimental data points in Fig. 4C where both factors are changed simultaneously but in a constant NaOH-to-$K_3Fe(CN)_6$ molar ratio of 10^4. Note that in the low concentration range the initial slope is decreasing from Thc to ThcPP. From these experiments optimal concentration values of 1.54×10^{-4} and 1.44 *M* were adopted for $K_3Fe(CN)_6$ and NaOH, respectively. These concentrations are indicated by arrows in the figures.

Methanol was mixed in increasing proportion with the mixture of thiamines before the oxidation (Fig. 5). Within a range of 10—50% methanol, retention times were perfectly stable but a linear increase in peak area was observed for each compound. This allows a 20% increase in sensitivity when the sample is in

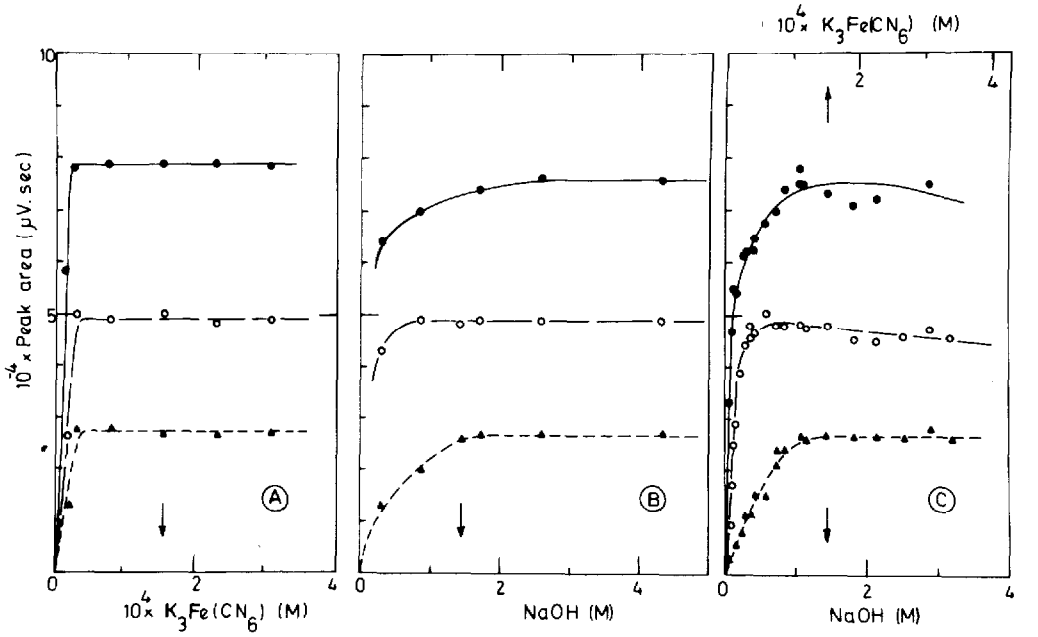

Fig. 4. Effect of $K_3Fe(CN)_6$ (A), NaOH (B) and combined reagents (C) on the oxidation reaction as measured by the peak areas of chromatograms obtained in gradient mode (see Fig. 2). The arrows indicate the optimal concentration adopted in the precolumn derivatization procedure: 1.54×10^{-4} *M* for $K_3Fe(CN)_6$ and 1.44 *M* for NaOH. (•), Thc; (○), ThcMP; (▲), ThcPP.

50% methanol leading to a final methanol concentration of 31% of the oxidized sample, as described in the derivatization procedure section. Higher methanol concentrations were not assayed in order to avoid a decrease of retention which can occur if the sample is dissolved in a medium of eluting strength higher than that of the mobile phase. The observed enhancement of 20% of the oxidation yield agrees with the data of Wostmann and Knight for thiamine [21]. In addition to the regulation of retention, methanol is thus advantageously used here to optimize the precolumn reaction.

In the above experiments, the mixture of thiamines was oxidized exactly 1 min before the injection. A reduction of the reaction time to 30 sec or an increase up to three days did not affect the chromatographic results. The derivatives were stable when stored in the dark at room temperature which allows the use of an autosampler for the analysis. Oxidized samples kept at −20°C in sealed vials were found to be unmodified three months after their preparation. This permits stable reference values for the analysis of biological samples.

Influence of sample composition and number on retention

Fig. 6 shows retention measurements for the experiments described in the previous section (Fig. 4). While changes in $K_3Fe(CN)_6$ concentration in the sample do not affect retention (Fig. 6A), the NaOH concentration has a significant influence on the retention of the phosphate derivatives (Fig. 6B and C). The magnitude of the effect seems to be related to the number of charged

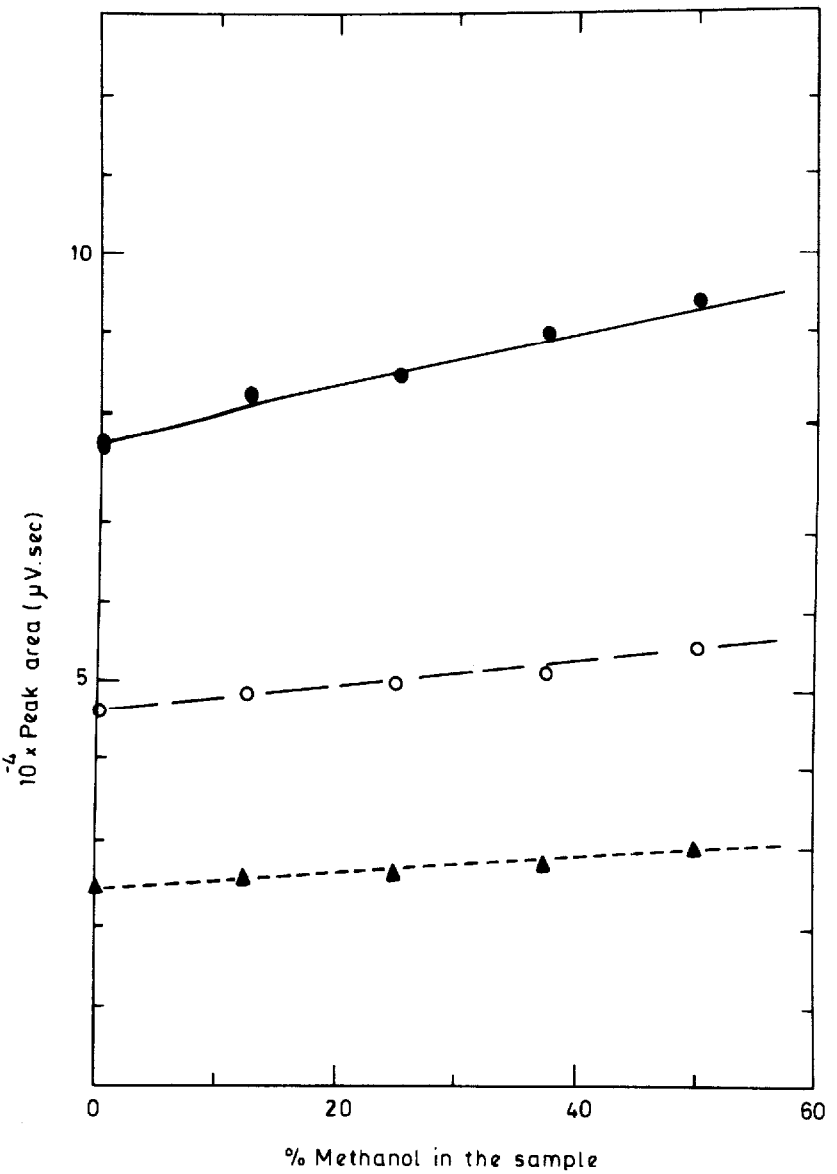

Fig. 5. Role of methanol on the oxidation reaction. ThcPP (▲), ThcMP (○) and Thc (●) were eluted at 4.56, 5.16 and 6.75 min, respectively, for each experimental condition.

phosphate groups. For NaOH concentrations equal or higher to the selected value (1.44 *M*) constant retention times are obtained together with a maximum selectivity.

The number of samples, injected at the same 14-min time interval and derivatized under optimal conditions, also affects the same chromatographic parameters, retention and selectivity, as shown in Fig. 7. The amplitude of the effect, however, is less pronounced than that described above for NaOH. In practice, two preliminary injections are required to obtain stable conditions. To shorten this stabilization time, three successives injections at 30-sec intervals could be made before starting quantitative determinations.

Such an influence of sample composition and number on chromatographic

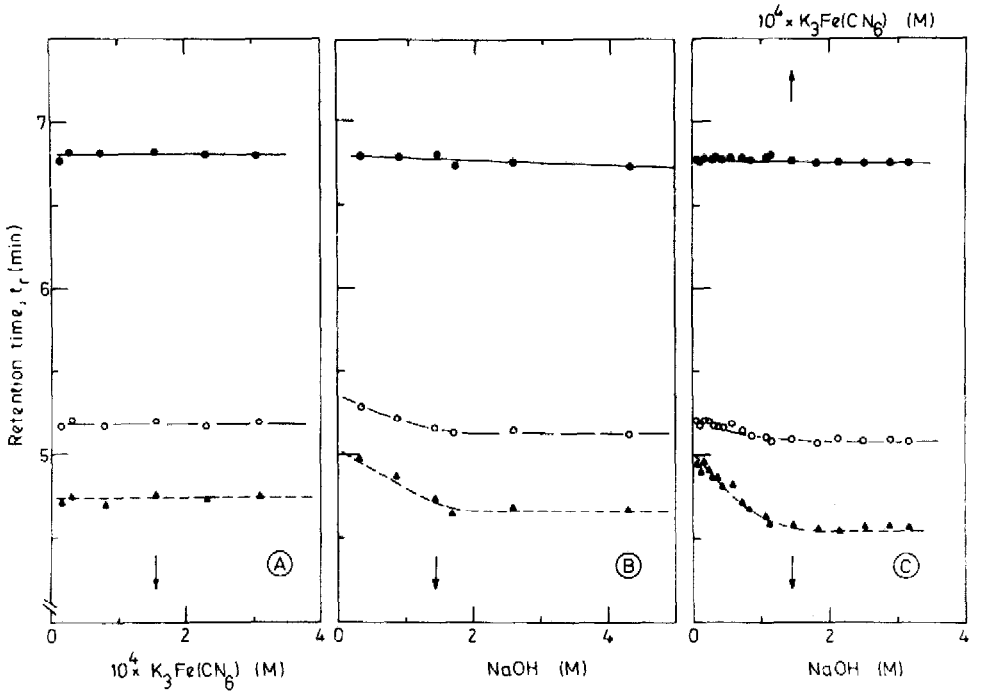

Fig. 6. Influence of the concentration of $K_3Fe(CN)_6$ (A), NaOH (B) and combined reagents (C) on the retention time of thiochrome derivatives (t_0 = 1.4 min). The experimental conditions are identical to those of Fig. 4. (●), Thc; (○), ThcMP; (▲), ThcPP.

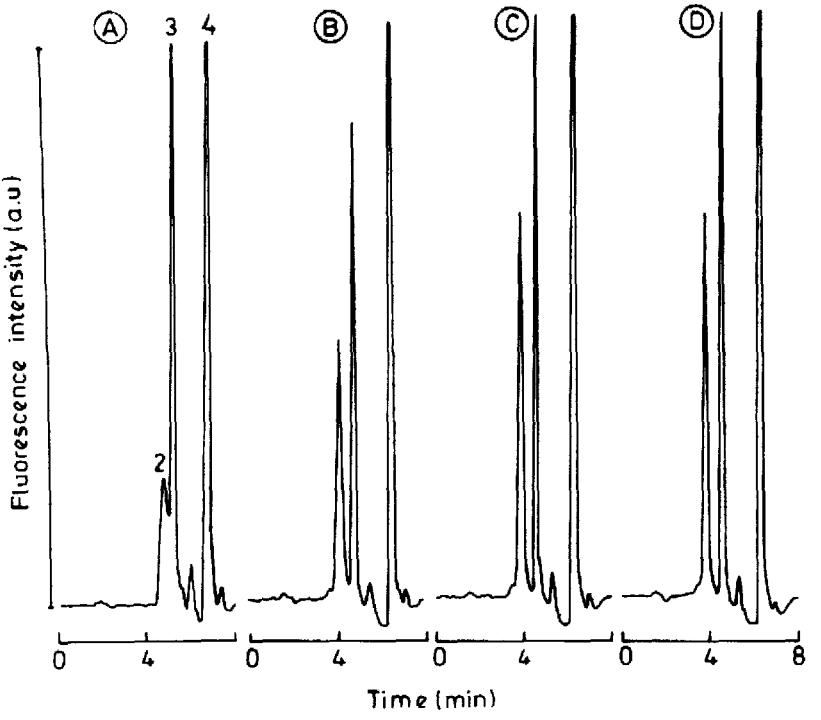

Fig. 7. Stabilization of the chromatographic system by repeated injection of an oxidized standard mixture of TPP (peak 2), TMP (peak 3) and T (peak 4).

behaviour should of course be avoided whenever possible in analytical techniques. However, highly reproducible results can be obtained if the time between successive injections is constant.

Column stability

The efficiency of a new column was maintained for one to two months in spite of intensive use (8 h per day) with an alkaline buffer (pH 8.4) as mobile-phase component and the injection of samples containing 1.44 *M* NaOH. The lifetime of the analytical column was not significantly increased by the use of precolumns. This drawback is not reported by the users of such an alkaline mobile phase for the analysis of thiamines [15, 16, 19].

Such a high pH was initially selected to obtain the best fluorescence intensity of the thiochromes [15]. Attempts were made to lower the pH of the buffer but resulted in a significant loss in sensitivity.

Quantitative analysis

A linear response was obtained over a large range, 0.1—20 pmol, with the same detection limit of 0.05 pmol reached by Sanemori et al. [16] who used cyanogen bromide as oxidant and 2.5% DMF as the organic mobile-phase component. It is worth noting that Thc gives a higher fluorescence signal compared to ThcMP and ThcPP. This results from an effect of methanol on the fluorescence quantum yield of the thiochrome during the gradient elution. Indeed, equimolar amounts of thiamine and its phosphate esters produce equal thiochrome fluorescence values [22] as we have also observed under isocratic elution conditions.

The concentration of thiamine and of its phosphorylated derivatives was measured in samples related to the study of the relationship between thiamine compounds and the sodium-channel activity. Additionally, rat tissues were assayed for comparison with the data in the literature [19, 23].

Fig. 8A shows a typical chromatogram obtained with a sample of sciatic nerve from *Rattus norvegicus*. The total thiamine content was equal to 3.4 pmol/mg wet weight, with the following distribution: 3% of TTP, 70% of TPP, 13% of TMP and 14% of T. Similar chromatograms were registered with membrane extracts prepared from electroplaxes of *Electrophorus electricus*. This tissue is widely used as starting material for the purification of the sodium-channel proteins [9, 24].

When the method was applied to the analysis of rat tissues other than the sciatic nerve, TTP, which only accounts for 1% of the thiamines, could not be quantified. In the chromatogram presented in Fig. 8B, the peak of TTP is masked by a non-thiochrome peak. The amount of TPP + TMP + T in these tissues was found to be 6.85, 14.0 and 8.1 pmol/mg wet weight in brain, heart and kidney, respectively. These results are in agreement with those of Ishii et al. [19] using HPLC and of Rindi and De Giuseppe [23] using a low-pressure technique. The first authors noted that approximately 20% of the samples did not show TTP peaks under the best chromatographic conditions. For this reason, they developed another HPLC separation for the analysis of thiamine phosphates without thiamine [16]. In our case, higher retention is required for the identification of TTP. Attempts to increase retention by reducing methanol

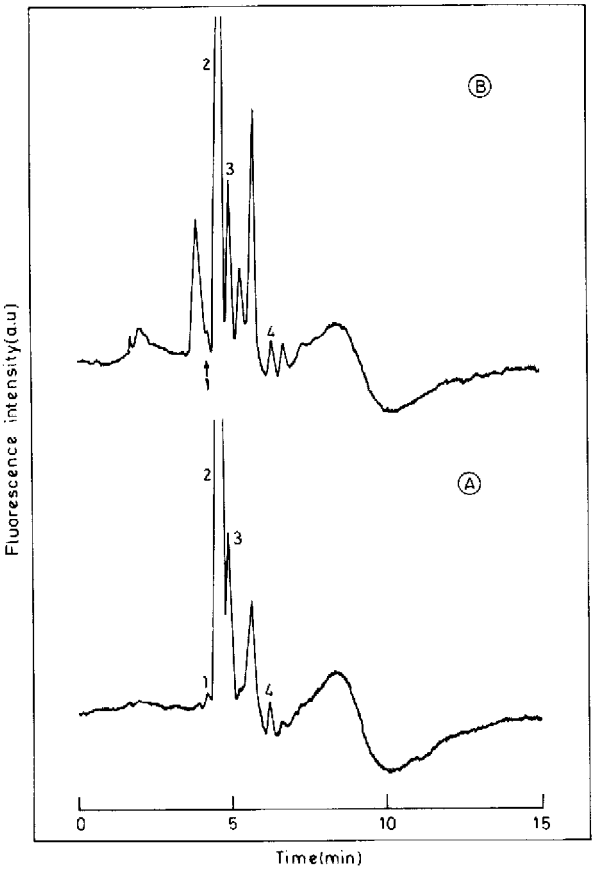

Fig. 8. Typical chromatograms of the analysis of rat sciatic nerve (A) and heart (B).

concentration gave rise to severe peak deformation (Fig. 2). Preliminary experiments have indicated that good peak symmetry can be obtained at low methanol concentration by using poly(styrene-divinylbenzene) instead of octadecyl silica. This should give more favourable conditions for the determination of TTP in such crude biological samples.

CONCLUSIONS

The results presented in this report clearly show that the oxidation of thiamine and thiamine phosphates by $K_3Fe(CN)_6$ can be used as a precolumn derivatization procedure instead of cyanogen bromide to reach the same order of sensitivity in chromatographic analysis. Moreover, the stability of $K_3Fe(CN)_6$ in NaOH allows the addition of the mixed reagents to the sample and this leads to a simpler and more accurate derivatization technique. The different parameters that affect the oxidation yield have been optimized. Among these factors, NaOH has been found to influence the chromatographic behaviour which requires carefully controlled analytical conditions: (1) a preliminary gradient cycle without injection; (2) three injections of oxidized standards at short intervals (30 sec); and (3) a constant time of 14 min between the sample injec-

tions. The role of NaOH seems to be related to the nature of the silica-based solid phase since it has not been observed in preliminary experiments on a poly-(styrene-divinylbenzene) column.

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