Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 42 (2006) 136–142
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Optimization of HPLC chromatographic conditions for determination of Transkarbam 12 and its degradation products

I. Pasáková [a,*], J. Klimeš [a], J. Sochor [a], A. Hrabálek [b]

[a] *Department of Pharmaceutical Chemistry and Drug Control, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic*
[b] *Department of Inorganic and Organic Chemistry, Charles University in Prague, Faculty of Pharmacy in Hradec Králové, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic*

Received 25 October 2005; received in revised form 9 March 2006; accepted 14 March 2006
Available online 22 May 2006

Abstract

This paper deals with searching of HPLC chromatographic conditions for determination and separation of Transkarbam 12 (T 12) and its two main degradation products (ω-aminocaproic acid and dodecylalcohol). T 12 is a new substance which belongs to the group of accelerators of transdermal penetration. Chromatographic separation was achieved using Separon SGX C18 analytical column (150 mm × 3 mm i.d.; 5 μm). Mobile phase contained acetonitrile and sodium acetate buffer pH 4.5 at the flow rate of 1 ml/min. Separation was carried out under the conditions of gradient elution. After the modification of the structure by derivatization reagent (3,5-dinitrobenzoyl chloride) detection at wavelength 230 nm was realized. The aim of this study was not only the optimization of the separation of derivatization reagent and derivatized T 12, Ak and D but also optimal derivatization processes for all three substances.

Keywords: Transkarbam 12; Transdermal penetration; 6-aminocaproic acid; Dodecylalcohol; RP-HPLC; Derivatization

1. Introduction

Transkarbam 12 (T 12) is a new substance which was synthesized in our Faculty at the Department of Inorganic and Organic Chemistry. The structure of the substance at room temperature was confirmed by infrared, ^{1}H and ^{13}C NMR spectra, as well as by elemental analysis and mass spectrometry [1]. T 12 is 5-(dodecyloxycarbonyl)pentylammonium-5-(dodecyloxycarbonyl)pentylcarbamate (see Fig. 1). It is a white or yellowish microcrystallic substance slightly dissolvable in water and most of organic solvents. The relationships between structure and activity of amphiphilic permeation enhancers with regard to their possible mechanisms of action were described by Vávrová et al. [2]. To elucidate the thermotropic phase behaviour of T 12, common methods were used, such as differential scanning calorimetry (DSC), FT-Raman spectroscopy, FT-infrared spectroscopy (FT-IR), X-ray powder diffraction, and dielectric relaxation spectroscopy [3].

T 12 belongs to a group of accelerants of transdermal penetration or also called permeation enhancers. In recent years transdermal drug delivery is more using for new approach to controlled drug administration [4]. The effect of these substances is based on temporarily modification of the skin barrier properties and after it causes better penetration of drugs to an organism. So they are used in cases when drugs at normal conditions do not pass through the skin barrier.

There are some important advantages against the other types of applications as per-oral or injective. It is mainly avoidance of first-pass metabolism by the liver which can in same cases change a drug to an inactive form in a high percentage. Then the doses of the substances must be much higher and it is more encumbering for an organism. Another advantage is a reduction of side effects and reduction of fluctuations of drug concentration in blood [4]. They are widely use in cases of adverse effects to gastrointestinal tract.

The structure of T 12 shows that there is a possibility to generation of hydrolytic degradation products → ω-aminocaproic acid and dodecylalcohol (Fig. 1). These two substances are also the starting substances for synthesis of Transkarbam 12 and therefore this study was aimed to these two. There exist no ana-

* Corresponding author. Tel.: +420 495 067 236; fax: +420 495 512 423.
E-mail address: ivana.pasakova@faf.cuni.cz (I. Pasáková).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.03.014

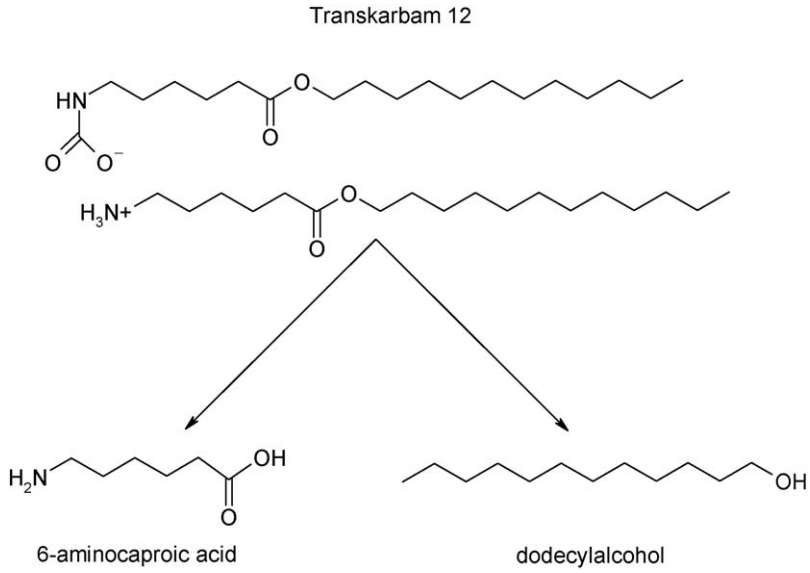


Fig. 1. Figures of the structure of Transkarbam 12 and its degradation products—dodecylalcohol and ω-aminocaproic acid.

lytical study about T 12 and its degradation products. Because of absence of any chromophore in the structures of substances derivatization processes were needed [5,6]. Thus the aim of this study was to develop optimal HPLC chromatographic conditions in which all three substances and derivatization reagent would be separated. Also derivatization processes for all three substances had to be developed.

2. Experimental

2.1. Chemicals

The standard of Transkarbam 12 (T 12) and standards derivatized by 3,5-dinitrobenzoyl chloride of T 12, Ak and D (D–T 12, D–Ak and D–D) were synthesized at the Department of Inorganic and Organic Chemistry at our Faculty and their structures were confirmed. Standards of ω-aminocaproic acid (Ak) and dodecylalcohol (D) were obtained from Sigma-Aldrich (Czech Republic). Acetonitrile, methanol, acetic acid, sodium acetate buffer and chloroform were obtained from Lachema a.s. (Brno, Czech Republic). Derivatization reagent 3,5-dinitrobenzoyl chloride (3,5-DNBC) and triethylamine (TEA) were purchased from Fluka (Sigma-Aldrich, Czech Republic). The water was purified using reverse osmosis.

2.2. Chromatographic conditions

2.2.1. Apparatus

The isocratic analyses were recorded on a chromatographic system (Thermo Separation Products Inc., Riviera Beach, USA) which consisted of an isocratic pump (Costametric 3500), an autosampler (AS 1000) and a UV–vis detector (UV 3000 HR). The HPLC data were processed with SpectraSystem Software-PC 1000 operated under the control of OS/2 Warp operation system.

The gradient analyses were recorded on a chromatographic system series HP 1100 (Agilent Technologies, Palo Alto, USA) which consisted of a HP 1100 series binary pumping system, a vacuum degasser, a thermostated column compartment, a variable wavelength detector and an autosampler. The chromatographic data using HP Chemstation (Agilent technologies) were processed.

UV–vis Spectrophotometer UV-2401 PC (Shimadzu, Duisburg, Germany) was used for measuring UV spectra of the substances. The results on PC with UVPC-Personal Spectroscopy Software were compiled.

2.2.2. HPLC conditions

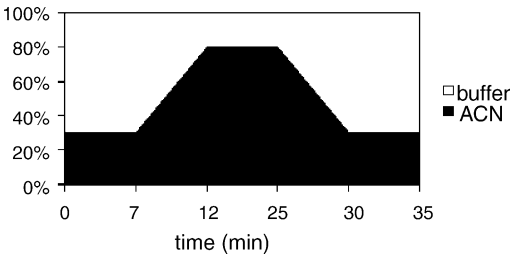
Separations for isocratic and also for gradient elution were performed on a Separon SGX C18 analytical column (150 mm × 3 mm i.d.; 5 μm particle size) and kept at 25 °C. The flow rate was 1 ml/min in all experiments and 20 μl sample volumes into the chromatographic system were injected. Wavelength used for the detection was 230 nm.

Isocratic elution: Mobile phases for isocratic elution were composed of mixture of acetonitrile and water in various ratios. The mobile phase with acetonitrile and 1% aqueous acetic acid was also tried in different ratios.

Gradient elution: The final mobile phase for gradient elution contained acetonitrile and 20 mM sodium acetate buffer pH 4.5 (adjusted with 10% acetic acid).

Gradient scheme of the analysis

Time (min)	20 mM sodium acetate buffer, pH 4.5 (%)	Acetonitrile (%)
0–7	70	30
7–12	70 → 20	30 → 80
12–25	20	80
25–30	20 → 70	80 → 30
30–35	70	30



2.2.3. Sample preparation

All artificially derivatized standards samples—D–T 12, D–Ak, D–D were prepared by dissolving in the mobile phase in concentration cca. 1 mg/ml and then analysed by HPLC.

2.3. Derivatization process

2.3.1. Derivatization of Transkarbam 12 and dodecylalcohol

The mixture of 1 mg of each standard substance (T 12 and D) was dissolved in 3.0 ml of chloroform and 0.2 ml of TEA and

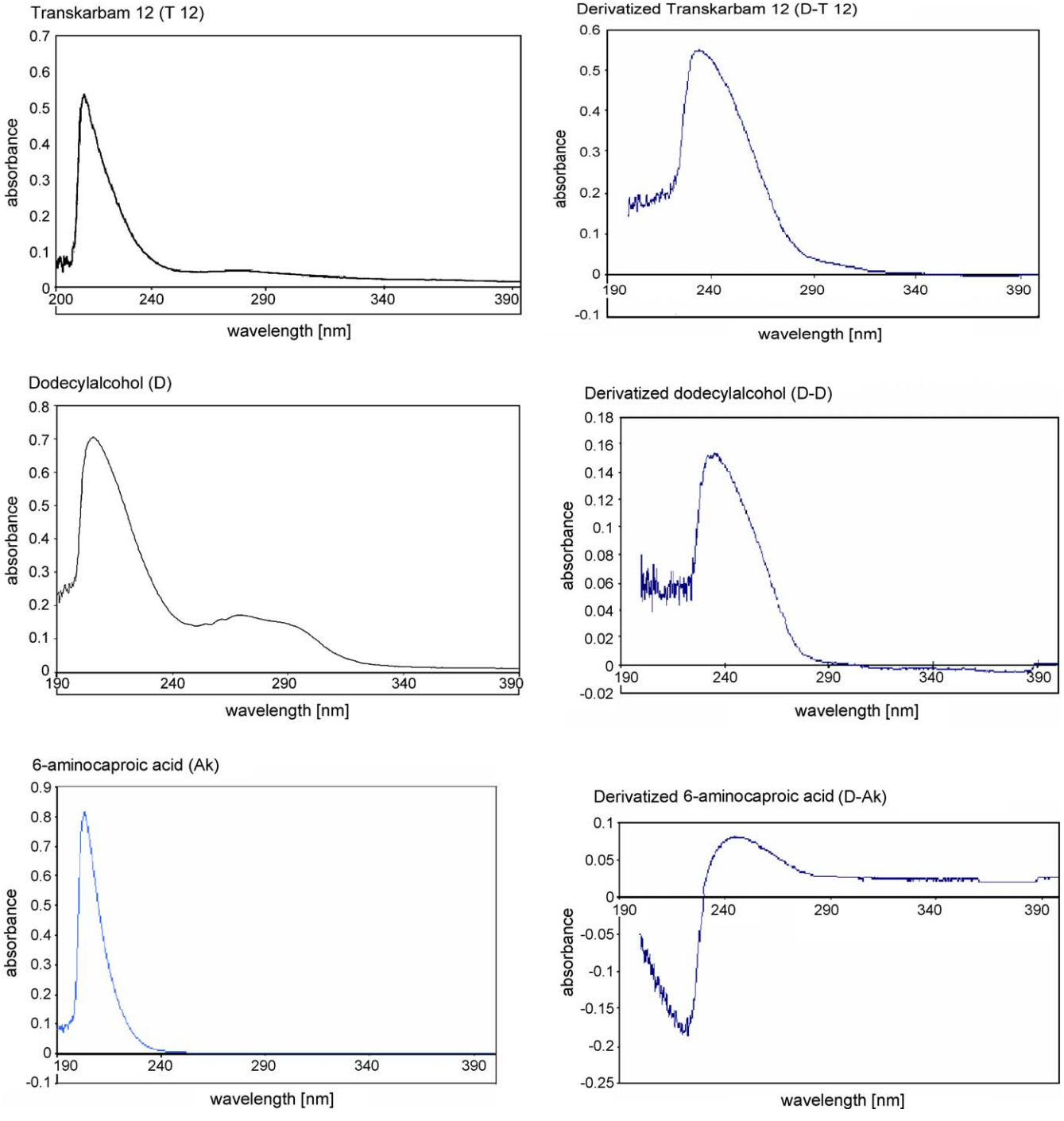


Fig. 2. Differences between UV-spectra of standards of Transkarbam 12, ω-aminocaproic acid, dodecylalcohol and their standards of derivatized substances.

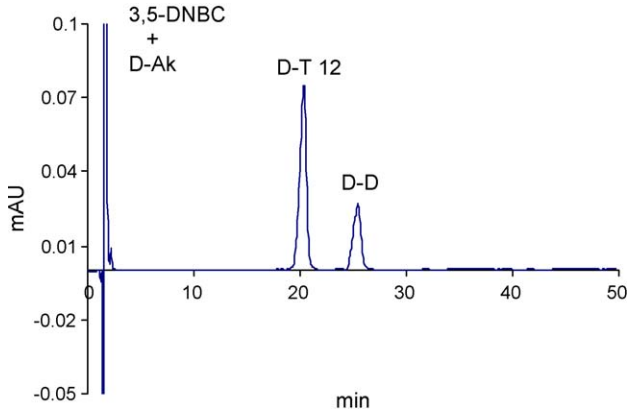


Fig. 3. Chromatogram of the separation of 3,5-DNBC; D–Ak; D–T 12 and D–D under isocratic conditions with mobile phase consisting of acetonitrile–water–acetic acid in a ration 80:20:1 (v/v/v). The flow rate was 1 ml/min.

after that 20.0 mg of 3,5-DNBC were added. Heating in a water bath at 65 °C for 30 min followed. In the end the sample was evaporated to dryness by nitrogen stream and then dissolved in 1 ml of mobile phase and analysed by HPLC.

2.3.2. Derivatization of ω-aminocaproic acid

The principle of the derivatization of Ak was the same as for T 12 and D. One milligram of the substance was dissolved—the only difference was another used solvent. Methanol was used in place of chloroform.

3. Results and discussion

3.1. UV-spectra

HPLC analysis of Transkarbam 12 in itself was not possible because of absence of chromophore in its structure. Also its degradation products (Ak and D) have no chromophore. The absorbance of T 12, D and Ak were about 210 nm. The derivatization using 3,5-dinitrobenzoyl chloride (3,5-DNBC) was carried out for better detection limits of all three substances in the UV-area because at this wavelength absorb a lot of others substances. The absorption maximum for derivatized T 12 (D–T 12) was shifted to 230 nm. Absorbance of derivatized degradation products were also about 230 nm. At this wavelength all compounds studied show a sufficient absorption. All spectra of non-derivatized and derivatized substances are shown at Fig. 2.

3.2. Isocratic or gradient elution

As the first step of optimization of conditions for separation (3,5-DNBC, D–T 12, D–Ak and D–D) isocratic elution was chosen. Mobile phase containing acetonitrile and water or 1% aqueous acetic acid in the ratio 80:20 (v/v) was tried. Separation of D–T 12 and D–D was satisfactory under these conditions but the detection of D–Ak was not possible. 3,5-DNBC and D–Ak had the identical retention time and they co-eluted around second minute (Fig. 3). Therefore other ratios of the component of the mobile phase for better separation of these two substances were tested. It was supposed that less amount of acetonitrile evoked stronger retention of D–Ak on the column. Thus more aqueous mobile phase 30:70 (v/v) was tried. Prolongation of the retention time of D–Ak and its separation from 3,5-DNBC was reached with this mobile phase but problem was with the detection of D–T 12 and D–D. Because these substances are hydrophobic there was problem with their elution. After 1 h there was no peak of these substances on the chromatogram. D–Ak needed for the separation more aqueous mobile phase but these conditions were not suitable for D–T 12 and D–D which needed more organic mobile phase. Because of these noticeable differences among substances isocratic elution was not possible and gradient elution was used. The ratio of 30% of acetonitrile (ACN) and 70% of water was better for the separation of the 3,5-DNBC and D–Ak. Mobile phase containing 80% ACN and 20% water was better for the detection of the D–T 12 and D–D. Therefore the separation of these substances was provided under the conditions of the gradient elution. More time schemata of the analysis were tried. As the final following one was chosen—0–7 min: 30% (ACN); 7–12 min: 30 → 80% (ACN); 12–25 min: 80% (ACN); 25–30 min: 80 → 30% (ACN); 30–35 min: 30% (ACN).

3.3. Mobile phase

Another step was to optimize the composition of the mobile phase for gradient elution. Various types of mobile phases, ratios and values of pH of the aqueous component for the optimization of the mobile phase were tested and the results were compared.

3.3.1. ACN/water with acetic acid

As the first type of mobile phase for gradient elution acetonitrile and water with various volumes of acetic acid was tried. The test of influence five different concentrations of acetic acid in the water was accomplished. Aqueous solutions containing 1%; 0.5%; 0.1%; 0.05% and 0% of acetic acid were tested (Fig. 4).

The differences between retention times of 3,5-DNBC and D–Ak were marked with changes of volume of the acetic acid in the solution. At most the amount of acetic acid influenced the retention of 3,5-DNBC.The bigger volumes 1% or 0.5% in contrast to 0.1% acetic acid in aqueous part of mobile phase evoked prolongation of the retention time of 3,5-DNBC (from R_t = 1.7 min at 0.1% acetic acid to R_t = 3.4 min at 1% acetic acid). But the retention time of D–Ak was not affected so much (from R_t = 7.4 min at 0.1% acetic acid to R_t = 7.1 min at 1% acetic acid). The higher concentrations of the acetic acid caused approximation of the retention times of 3,5-DNBC and D–Ak. The influence on the retention times of the other substances was not so perceptible. Contrariwise the smaller volumes (0.05% or 0%) were appreciated for the separation of 3,5-DNBC and D–Ak which was relatively suitable. But the separation of the D–T 12 and D–D was insufficient.

The best results were achieved with mobile phase with content of 0.1% acetic acid. The separation of the derivatized standard of T 12 and its degradation products was optimal. But when the derivatization process in itself was tried an interference with blank of derivatization mixture was evident (see Fig. 5). Thus this type of the mobile phase was not suitable for analysis of

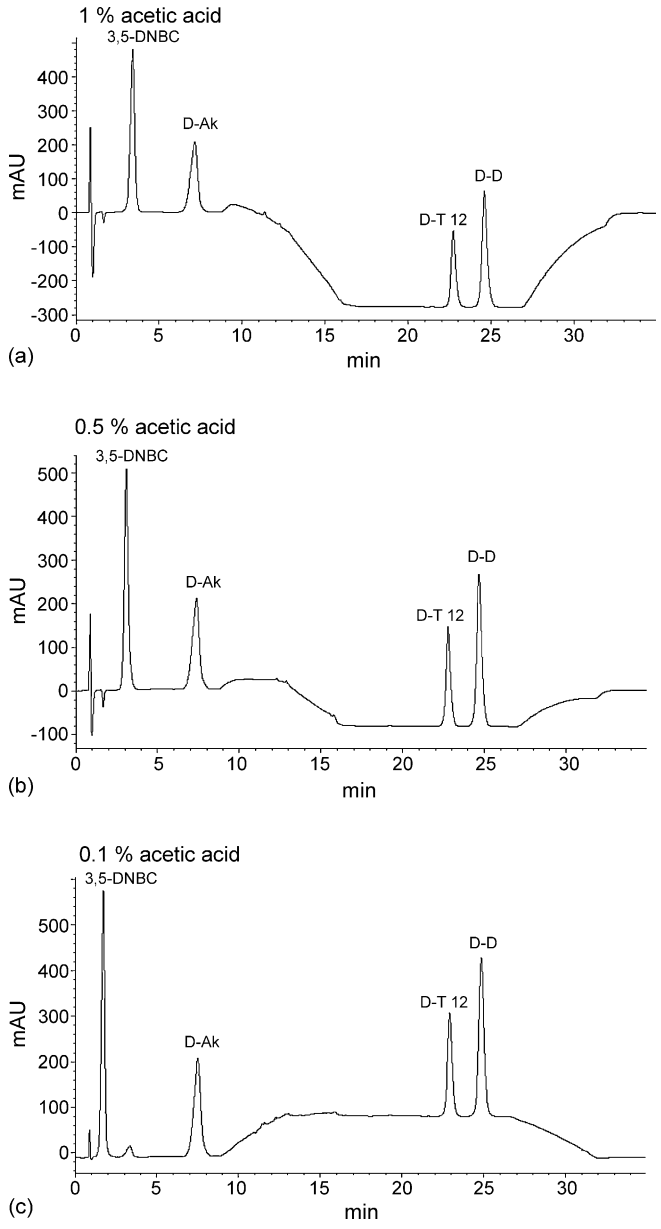


Fig. 4. Different concentrations of acetic acid in the aqueous part of mobile phase for separation of the standards of derivatized substances: (a) 1% acetic acid, (b) 0.5% acetic acid and (c) 0.1% acetic acid. This measurement proceeded under optimal conditions—gradient scheme was—0–7 min: 30% ACN; 7–12 min: 30 → 80% ACN; 12–25 min: 80% ACN; 25–30 min: 80 → 30% ACN; 30–35 min: 30% ACN. Separon SGX C18 analytical column (150 mm × 3 mm i.d.; 5 μm) was used and kept at 25 °C. The flow rate was 1 ml/min.

the real samples. Peaks between 10th and 13th minute which proceeded from the derivatization reagent were also visible on the chromatogram. They were situated in a suitable retention time—no peaks of studied substances are eluated at this time.

3.3.2. ACN/20 mM sodium acetate buffer

The second type of mobile phase containing sodium acetate buffer was tried. The changes of the values of pH were tested because it was supposed that it influenced retention time of D–Ak. Three pH values were examined—pH 4.5, 5.2 and 6.0 (Fig. 6).

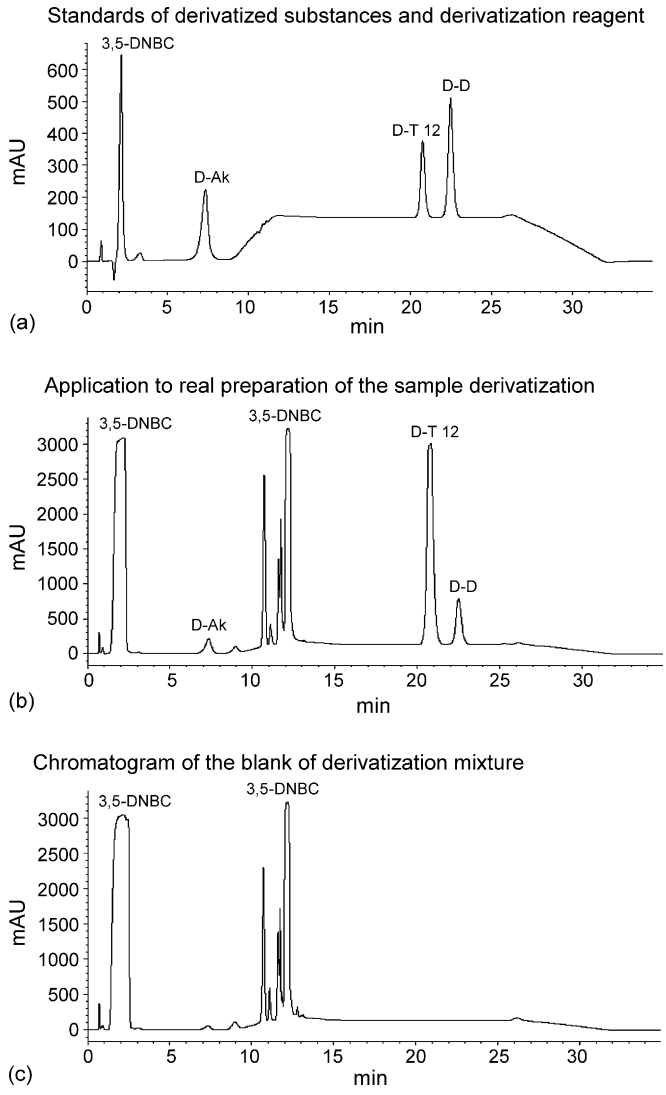


Fig. 5. Application of the first type of optimal mobile phase containing 0.1% acetic acid to: (a) the standards of derivatized substances and derivatized reagent (D–Ak, D–T 12 and D–D and 3,5-DNBC), (b) application to real preparative of derivatization of the sample, and (c) chromatogram of blank of derivatization mixture containing chloroform, TEA and derivatization reagent. Gradient scheme was—0–7 min: 30% ACN; 7–12 min: 30 → 80% ACN; 12–25 min: 80% ACN; 25–30 min: 80 → 30% ACN; 30–35 min: 30% ACN. Separon SGX C18 analytical column (150 mm × 3 mm i.d.; 5 μm) was used and kept at 25 °C. The flow rate was 1 ml/min.

The retention time of D–Ak decreased with increasing values of pH. Retention time of D–Ak at pH 6.0 was R_t = 1.5 min and for 3,5-DNBC was R_t = 0.8 min. At pH 5.2 was R_t (D–Ak) = 3.1 min and R_t (3,5-DNBC) = 0.9 min Whence it follows that the separation of the 3,5-DNBC and D–Ak was not sufficient when pH 6.0 or 5.2 were tested. The optimal resolution of 3,5-DNBC, D–Ak and also D–T 12 and D–D with pH 4.5 was achieved. At pH 4.5 following retention times were measured: R_t (3,5-DNBC) = 0.9 min, R_t (D–Ak) = 5.4 min, R_t (D–T 12) = 20.0 min and R_t (D–D) = 21.5 min. Thus modifications of pH had the biggest influence to the retention time of D–Ak. The retention times of others three substances (3,5-DNBC, D–T 12 and D–D) were not influenced by different values of pH of the aqueous part of the mobile phase. Longer distance between peaks of D–Ak

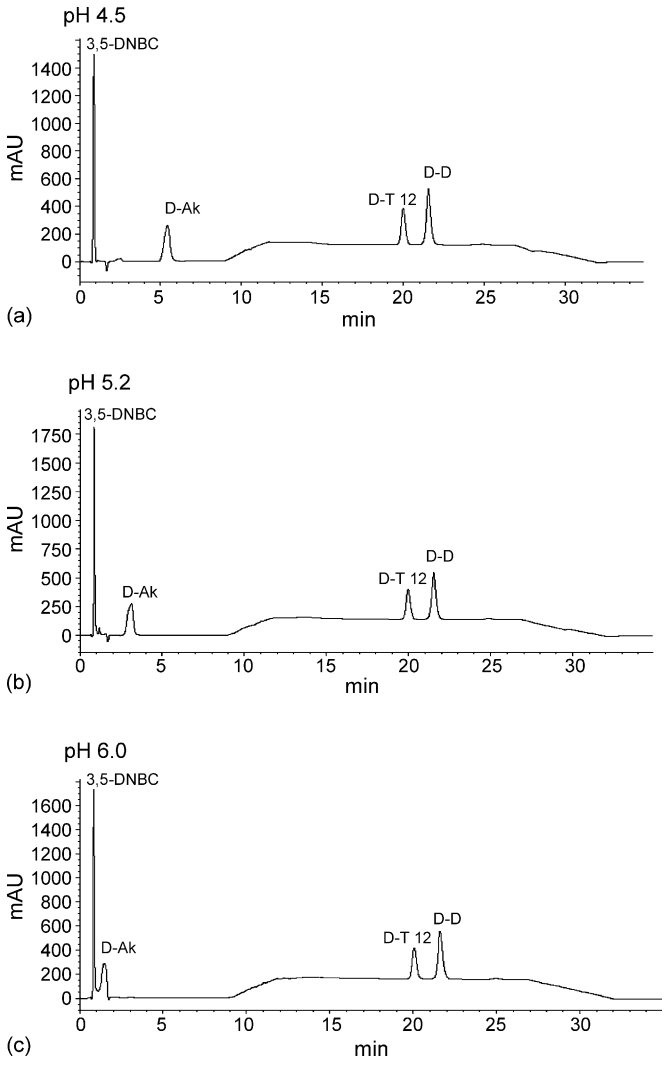


Fig. 6. Chromatograms of influence of three pH values of 20 mM sodium acetate buffer on the retention time of the standards of derivatized substances—3,5-DNBC, D–Ak, D–T 12 and D–D: (a) pH 4.5, (b) pH 5.2 and (c) pH 6.0. Gradient scheme was—0–7 min: 30% of acetonitrile; 7–12 min: 30→80% ACN; 12–25 min: 80% ACN; 25–30 min: 80 → 30% ACN; 30–35 min: 30% ACN. Separon SGX C18 analytical column (150 mm × 3 mm i.d.; 5 μm) was used and kept at 25 °C. The flow rate was 1 ml/min.

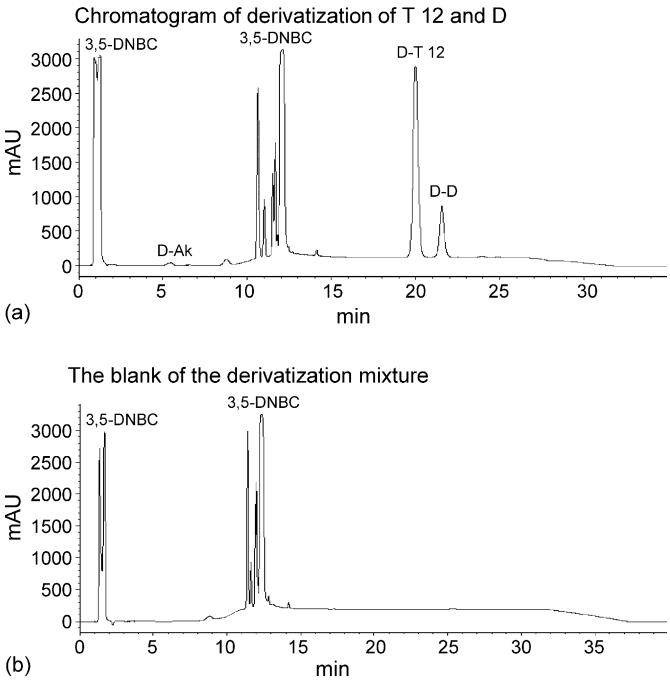


Fig. 7. Derivatization process of T 12 and D. (a) The ideal derivatization process for T 12 and D with chloroform, TEA and derivatization reagent. (b) The blank of the derivatization mixture containing chloroform, TEA and 3,5-DNBC. Chromatogram of D–T 12 and D–D proceeded under optimal conditions with mobile phase containing ACN and 20 mM sodium acetate buffer, pH 4.5. Gradient scheme—0–7 min: 30% ACN; 7–12 min: 30 → 80% ACN; 12–25 min: 80% ACN; 25–30 min: 80 → 30% ACN; 30–35 min: 30% ACN. Separon SGX C18 analytical column (150 mm × 3 mm i.d.; 5 μm) was used and kept at 25 °C. The flow rate was 1 ml/min.

and 3,5-DNBC was also needed because of excess of derivatization reagent during the preparation real samples. The excess of derivatization reagent in reactive mixture was necessary because of quantitative reaction.

3.4. Derivatization process

At first the derivatization process with 3 ml of chloroform, 0.2 ml of TEA and 20 mg of 3,5-DNBC was tried. This process was optimal for Transkarbam 12 and dodecylalcohol, but the derivatization of ω-aminocaproic acid was not sufficient.

Then influence of the amount of derivatization reagent was tested. An amount of 10, 20 and 40 mg of 3,5-DNBC were tried. But it had no influence on the quantity of the reaction and Ak was even with 40 mg of 3,5-DNBC still only slightly detectable. To find the optimal derivatization process for Ak was the next step. Ak is bad soluble in most of solvents and it dissolves only in water and methanol. Water was not possible to use because of derivatization reagent which becomes inactive in water. The process with 3 ml of methanol, 0.2 ml of TEA and 20 mg of 3,5-DNBC was used and the reaction was acceptable. So there was an endeavour to develop one step derivatization. But a problem appeared with derivatization of dodecylalcohol which was not realized when methanol as a dissolving solution for Ak was used**.** This fact is illustrated at Fig. 8a. Some amount of derivatized T 12 is visible but there is no peak of D. Different derivatization procedures were tested. They varied in the order of adding of the solutions or in the number of steps of derivatization. But no derivatization for dodecylalcohol has been found under these conditions. Also adding of chloroform, TEA and derivatization reagent (for derivatization of T 12 and D) was tried, heating and evaporating to dryness by nitrogen stream followed and then methanol, TEA and 3,5-DNBC were added for derivatization of Ak. Thus the derivatization procedure passed twice (once for T 12 and D and for the second time for Ak) but also this process was unsuccessful.

The differences among the structures were so big that it was not possible to find any process which could be used for all these substances. So the two types of derivatization were used. The sample had to be divided in two parts. The first part was derivatized with 3 ml of chloroform, 0.2 ml of TEA and 20 mg of 3,5-DNBC for derivatization of T 12 and D (Fig. 7a). The same

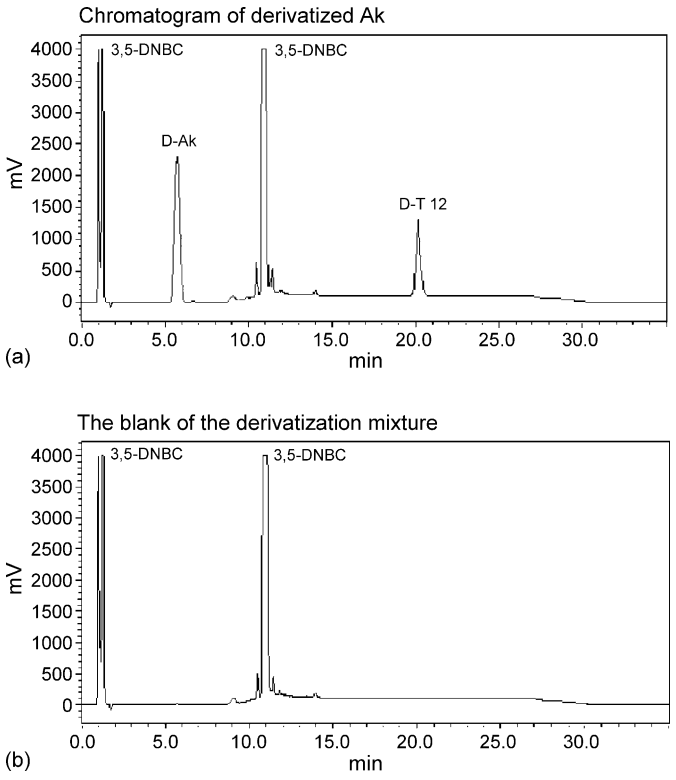


Fig. 8. Derivatization process of Ak. (a) The ideal derivatization process for Ak with methanol, TEA and 3,5-DNBC. (b) The blank of the derivatization mixture containing methanol, TEA and derivatization reagent. Chromatogram of derivatized Ak was proceeded under optimal conditions with mobile phase containing ACN and 20 mM sodium acetate buffer, pH 4.5. Gradient scheme and the flow rate were the same as at Fig. 7.

process of the sample preparation was tried with all solutions and derivatization reagent, only without the substances. This chromatogram of the blank is shown in Fig. 7b. And the second one containing 3 ml of methanol, 0.2 ml of TEA and 20 mg of 3,5-DNBC for derivatization of Ak.

To avoid these complicated two-procedure derivatizations of degradation products it would be possible to result from one theoretic solution relating to the symmetric structure of T 12.

This should be a way for using only the first type of derivatization where the amount of T 12 and D is measurable. Because these two degradation products (Ak and D) are generated in an equimolar amount so the amount of D–Ak should be kept count of the amount of dodecylalcohol.

4. Conclusions

Derivatization processes for all these substances had to be found for the determination of Transkarbam 12 and its two degradation products (dodecylalcohol and ω-aminocaproic acid). Two types of derivatization with 3,5-dinitrobenzoyl chloride as a derivatization reagent were developed. First one for T 12 and D and the second one for Ak.

After that optimization of chromatographic conditions for the separation of derivatization reagent and D–Ak, D–T 12 and D–D followed. A lot of different types of mobile phases were tested. The best results were achieved under gradient elution with mobile phase containing acetonitrile and 20 mM sodium acetate buffer pH 4.5. Wavelength used for the detection was 230 nm. Under these conditions separation of derivatization reagent and all three substances was carried out.

Acknowledgement

This work was supported by the research project of Ministry of Education, Youth and Sport of the Czech Republic No. 0021620822.

References

[1] A. Hrabálek, P. Doležal, O. Farsa, A. Krebs, A. Kroutil, M. Roman, Z. Škubalová, U.S. Patent 6,187,938 (2001).
[2] K. Vávrová, J. Zbytovská, A. Hrabálek, Curr. Med. Chem. 12 (2005) 2273–2291.
[3] J. Zbytovská, S. Raudenkolb, S. Wartewig, W. Hűbner, W. Rettig, P. Pissis, A. Hrabálek, P. Doležal, R. Neubert, Chem. Phys. Lipids 129 (2004) 97–109.
[4] N. Bűyűkzimkin, S. Bűyűkzimkin, J.H. Rytting, in: T.K. Ghosh, W.R. Phister (Eds.), Transdermal and Topical Drug Delivery System, Interpharm Press, Buffalo Grove, IL, 1997, pp. 357–367.
[5] E. Destandau, J. Vial, A. Jardy, M.C. Hennion, D. Bonnet, P. Lancelin, J. Chromatogr. A 1088 (2005) 49–56.
[6] K. Lemr, J. Ševčík, J. Hlaváč, J. Chromatogr. A 1021 (2003) 19–24.