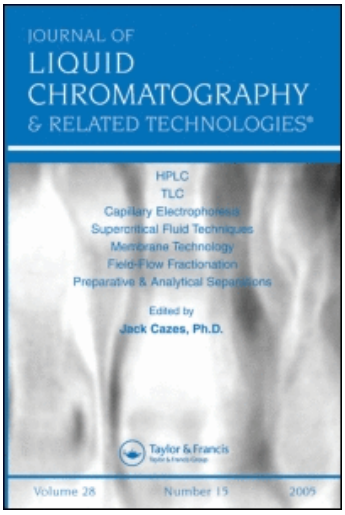

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 29 December 2009

To cite this Article Prukała, Dorota , Prukała, Wiesław , Szymalska, Magdalena , Witkowska-Krajewska, Ewa and Kluska, Mariusz(2010) 'OPTIMIZATION OF SEPARATION AND DETERMINATION OF HYDROXYSTILBAZOLE BENZYL DERIVATIVES BY ITP TECHNIQUE', Journal of Liquid Chromatography & Related Technologies, 33: 2, 250 — 258
To link to this Article: DOI: 10.1080/10826070903442287
URL: http://dx.doi.org/10.1080/10826070903442287

Journal of Liquid Chromatography & Related Technologies, 33:250–258, 2010

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903442287

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OPTIMIZATION OF SEPARATION AND DETERMINATION OF HYDROXYSTILBAZOLE BENZYL DERIVATIVES BY ITP TECHNIQUE

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□ *Benzyl derivatives of hydroxystilbazole show biological activity appearing as antimicrobial and anticancer effect. Moreover, these derivatives show properties of liquid crystals and can form complexes. In this paper are presented optimum conditions of separation and determination of two isomers, derivatives of hydroxystilbazole by a technique of isotachophoresis. (E)-N-(benzyl)-4′-hydroxystilbazole bromide and (E)-N-(benzyl)-2′-hydroxystilbazole bromides were subjected to an optimization process. Lengths of analysis steps, electric current intensity and pH of electrolyte solutions and samples were changed during the optimization of conditions. The shortest time of analysis was obtained during determination of individual isomers. However, in the case of mixtures, the time of analysis was lengthened two times and was at about 420 seconds. It was proven by a process of trial and error that optimum pH for analyzed isomers are 3.7.*

Keywords benzyl derivatives, bromides, hydroxystilbazole, isotachophoresis

INTRODUCTION

Investigations concerning biological activity of stilbene derivatives are carried out over the past dozens of years. The oldest investigations were involved in antimicrobial and insecticide activity of these compounds. They were started on the basis of observations of properties of different stilbene derivatives isolated from various species of plants.[1–3]

Already, at the beginning of the 1970s, 14 natural products related to compounds possessing stilbene structures, and their ethers and esters with carboxylic acids were known. These esters can be transformed by metabolic cyclization into corresponding coumarine derivatives.[4]

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Derivatives of stilbene occur in almost all species of pine, and in some species of eucalyptus and orange.[4] At the end of the XX century, stilbene derivatives were found in *Centipeda minima*,[5] *Cannabis sativa*,[6] *Chlorophora excelsa*,[7] and *Scirpus maritimus*.[8] Some of them have very complex structures as, for example, excelsa octaphenol (1,10-bis(3,5,2′,4′-tetrahydroxytransstilbenyl)-3,8-dimethyldeca-2,8-diene) described in 1989[7] or derivatives of rapontigenine isolated from *Rhizoma Rhei*.[9]

Great interest in this area of chemistry is caused by antimicrobial and anticancer activity and the fact that some complex compounds of (E)-stilbenes show liquid crystal properties. These interesting behaviors exhibit complexes with silver, molybdenum, and wolfram. During transformation from a solid state of aggregation into a liquid state, they form a liquid crystalline phase. Every one of the complexes of silver and wolfram described in the literature show temperature transformation from a crystalline phase to the smectic phase C, smectic phase A, and nematic phase, to isotropic phase. It enables further investigations and the use of these compounds in the resiliently developing electronic industry that takes advantage of liquid crystals.[10–12]

Appearance of biological activity of (E)-azastilbenes is well known. Transstyrylpyridines are effective inhibitors of choline acetyltransferase.[13–15] It was shown that N-substituted derivatives of (E)-4′-hydroxystilbazoles-4 exhibited antibacterial and fungistatic activity. Investigations were carried out testing following microrganisms: Gram-positive cocci (*Staphylococus aureus* 209P FDA, *Streptococus faecalis* ATCC 8040), aerobic bacilli (*Bacillus subtilis* ATCC 1633), Gram-negative rods (*Escherichia coli* PZH 026B6, *Klebsiella pneumoniae* 231, *Pseudomonas aeruginosa* SR_1), yeasts (*Candida albicans* PCM 1409 PZH), dermatophytes (*Microsporum gypseum* K_1) and moulds (*Aspergillus fumigatus* C_1).[14,15]

Optimization of a process for separation and determination of (E)-azastilbenes by various techniques, described in our work, certainly will be a contribution to the development of chemistry of these compounds. Until now, no attempts of determination of these compounds by isotachophoresis have been undertaken; no papers concerning this problem have been published. Therefore, in our work, an attempt has been taken to elaborate optimum conditions of separation and determination of chosen isomers, derivatives of hydroxystilbazole.

EXPERIMENTAL

Electrophoretic Analysis of Hydroxystilbazoles

Samples of isomers: bromide of (E)-N-(benzyl)-4′-hydroxystilbazole-4 and bromide of (E)-N-(benzyl)-2′-hydroxystilbazole-4 (Fig. 1) were dissolved

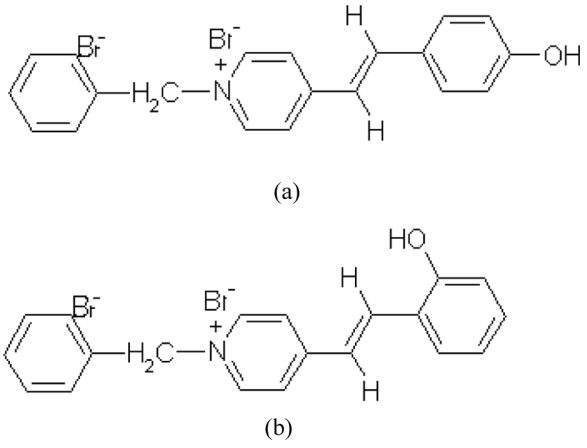

FIGURE 1 Structures of analyzed compounds (a) bromide of (E)-N-(benzyl)-4′-hydroxystilbazole-4 (**A4**), and (b) bromide of (E)-N-(benzyl)-2′-hydroxystilbazole-4 (**A6**).

in deionised water (Merck). In order to draw standard curves, we prepared standard solutions with concentrations: 2.0×10^{-3} mole L^{-1}, 5.0×10^{-4} mole L^{-1}, and 8.0×10^{-4} mole L^{-1}. Optimization of conditions of separation and determination were carried out by an ITP technique.

The method of optimization of analysis conditions of above mentioned compounds included preparation of solutions with known concentrations, obtaining standard curves for investigated compounds, and separation and determination of selected hydroxystilbazoles.

Electrolytes were prepared using reagents of analytical purity, namely, deionised water (Merck), hydrochloric acid (POCh Gliwice), acetic acid (POCh Gliwice), and sodium acetate (POCh Gliwice). Leading electrolytes

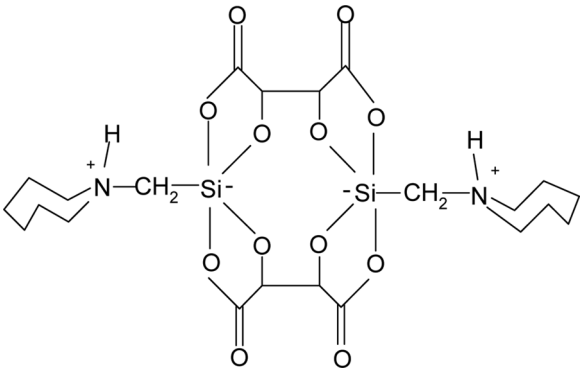

FIGURE 2 Structure of a compound used in terminating electrolyte: 4,4′-bis{1-(perhydroazepiniomethyl)[spirobi(1-sila-2,5-dioksacyklopentan-3-on)]at}.

TABLE 1 Chemical and Physical Data of Compounds[14]

Compound	Yield (%)	M.p. (°C)	IR (KBr) (cm^{-1}) $\delta_{CH=CH}$	^{1}H-NMR δ(ppm) $-CH_2-{}^+N$
A4	82.7	218–221	980	5.90
A6	87.8	247–249	955	5.86

Ld-1 and Ld-2 (pH = 3.7) were obtained by mixing of adequate volumes of solutions of hydrochloric acid and acetate buffer (from acetic acid and sodium acetate). As a terminating electrolyte (Tm) we used a solution of 4,4′-bis{1-(perhydroazepiniomethyl)[spirobi(1-sila-2,5-dioksacyklopentan-3-on)]at} with a concentration of 3.0×10^{-3} mole L^{-1} (Fig. 2).

The hydroxystilbazoles were prepared by the method described in the literature.[14] Selected chemical, physical and biological data are shown in Tables 1–2.

Apparatus

Optimization of separation and determination of the above mentioned derivatives of hydroxystilbazoles were carried out using a capillary electrophoresis analyzer, EA 202 M, produced by Villa Labeco (Slovakia), equipped with injection block with a container for terminating electrolyte, preseparation column (capillary diameter 0.8 mm, length 90 mm), bifurcation block with an electrode block of the preseparational column, analytical column (capillary diameter 0.3 mm, length 160 mm), and electrode block of the analytical column, UV detector, two conductometric detectors with a measurement range between 30 kΩ and 20 MΩ and steering unit – personal computer PC containing converter AD/DA.

^{1}H NMR spectra were recorded with a Bruker-200 in $CDCl_3$, with HMDS as internal standard. The infrared (IR) spectra were recorded with a Nicollet Magna-IR 760 in potassium bromide.

TABLE 2 Antimicrobial Activity of Isomers. Minimal Inhibitory Concentration (MIC $\mu g \cdot mL^{-1}$)[14]

	Minimal Inhibitory Concentration (MIC) $\mu g \cdot mL^{-1}$								
Compounds	1	2	3	4	5	6	7	8	9
A4	5	500	500	100	1000	1000	>500	>500	>500
A6	100	500	500	500	1000	1000	>500	>500	>500

1 - Staphylococcus aureus 209P FDA, 2 - Streptococcus faecalis ATCC 8040, 3 - Bacillus subtilis ATCC 1633, 4 - Escherichia coli PZHO 26B6, 5 - Klebsiella pneumoniae 231, 6 - Pseudomonas aeruginosa 5 R1, 7 - Candida albicanus PCM 1409 PZH, 8 - Microsporum gypseum K_1, 9 - Aspergillus fumigatus C1.

RESULTS AND DISCUSSION

Analyzed isomers belonging to the group of hydroxystilbazoles: (E)-N-(benzyl)-4′-hydroxystilbazole bromide and (E)-N-(benzyl)-2′-hydroxystilbazole bromide were investigated in order to separate and determine them in as short a time as possible. In the investigations we used isotachophoresis.

Optimum parameters of methods of separation and determination of individual isomers and their mixtures are presented in Tables 3–5; isotachophoregrams obtained in optimum conditions are shown in Figs. 3–5.

TABLE 3 Conditions of the Method of Individual Determination (E)-N-(benzyl)-4′-hydroxystilbazole-4 (**A4**)

	Considered Parameters			
Stage	Time [s]	Intensity [μA]	Comp [10 mV]	Conductometric Detector
1	100	80	0	
2	45	160	50	
3	60	50	0	X

TABLE 4 Conditions of the Method of Individual Determination Bromide of (E)-N-(benzyl)-2′-hydroxystilbazole-4 (**A6**)

	Considered Parameters			
Stage	Time [s]	Intensity [μA]	Comp [10 mV]	Conductometric Detector
1	100	80	0	
2	60	90	50	
3	60	50	0	X

TABLE 5 Optimum Conditions of Isotachophoretic Separation of a Mixture of Bromide of (E)-N-(benzyl)-4′-hydroxystilbazole-4 (**A4**), and Bromide of (E)-N-(benzyl)-2′-hydroxystilbazole-4 (**A6**)

	Considered Parameters			
Stage	Time [s]	Intensity [μA]	Comp [10 mV]	Conductometric Detector
1	100	100	0	
2	150	250	0	X
3	65	10	0	
4	10	130	0	
5	30	110	50	
6	100	35	0	X

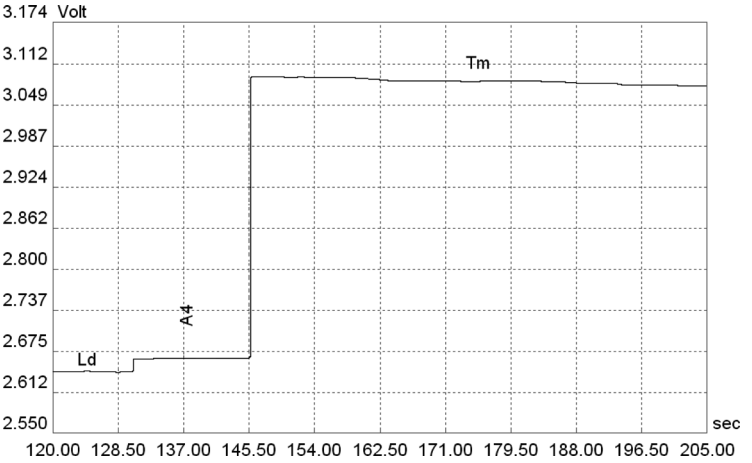

FIGURE 3 Isotachophoregram of (E)-N-(benzyl)-4′-hydroxystilbazole-4 (**A4**).

During a determination by the ITP technique, an electric field causes ions placed between a system of two electrolytes (leading and terminating) migrate towards adequate potential. Ions are moving according to their mobilities, in turn, from the highest to the lowest electrophoretic mobility. Ions of terminating electrolyte must have the lowest electrophoretic mobility.

During optimization of separation and determination of the above mentioned hydroxystilbazoles, different conditions of the method were tested. Time of analysis, intensity of the electric current, pH range (starting from zero to seven, because cation forms were analyzed), and level of limitation of high voltage were changed with the use of the preseparational column or a system of two columns (i.e., preseparational and analytical).

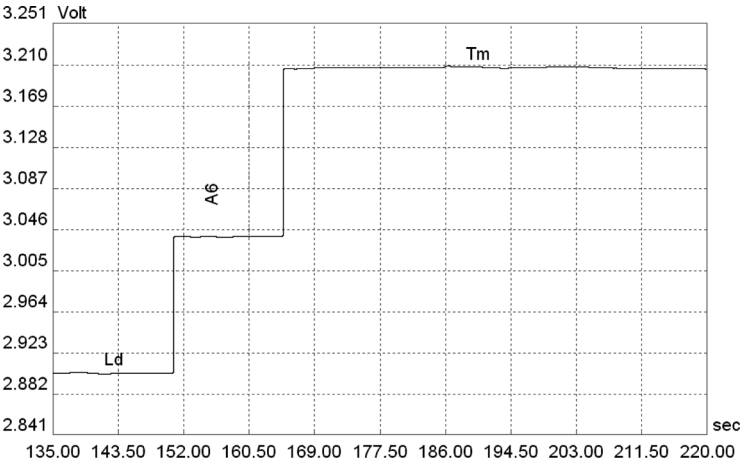

FIGURE 4 Isotachophoregram of bromide of (E)-N-(benzyl)-2′-hydroxystilbazole-4 (**A6**).

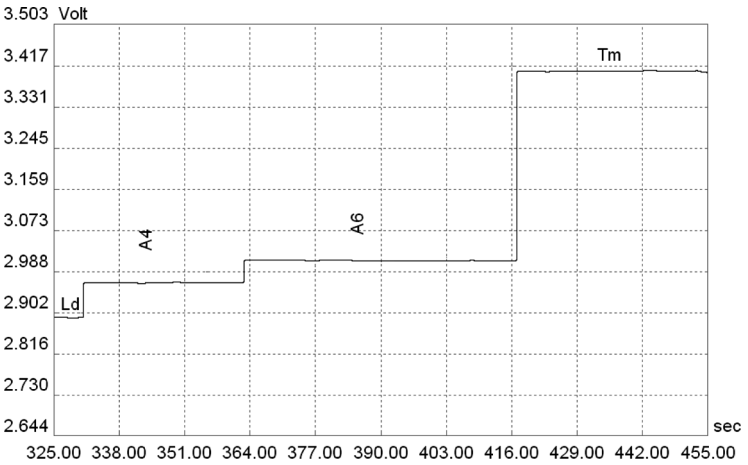

FIGURE 5 Isotachophoregram of the mixture of bromide of (E)-N-(benzyl)-4′-hydroxystilbazole-4 (**A4**), and bromide of (E)-N-(benzyl)-2′-hydroxystilbazole-4 (**A6**).

Analyses were carried out changing the voltage from 9 kV to 15 kV. At the voltage lower than 9 kV separation was not achieved. Similarly, no effects were obtained at the voltage higher than 12 kV.

Analysis of the above mentioned isomers was carried out in acidic solutions. During the analysis, only cations were determined. Anionic forms were not determined because bromide was the only anion. The separation was performed on the basis of differences of electrophoretic mobilities of analyzed cations. Analyzed compounds showed very similar electrophoretic mobilities. For the determinations, we used a solution of terminating electrolyte characterized by significantly lower mobility than analyzed isomers.

The terminating electrolyte for the analysis of investigated mixtures was selected by a process of trial and error. It consists of an aqueous solution of 4,4′-bis{1-(perhydroaminiomethyl)[spirobi(1-sila-2,5-dioxacyclopentan-3-on)]at}. This compound is a hypercoordinated organosilicon compound. Common conditions of analyzed heptacoordinated derivatives are shown in Table 5.

The separation of the mixture was difficult, because the compounds differed only by the position of the substituent in the ring and showed similar mobilities. During the optimization process, time of analysis, intensity of the electric current, and pH of solutions of leading electrolytes and samples were changed. By a process of trial and error, it was proven that the optimum pH for analyzed isomers was 3.7. At pH close to seven, isotachophoregram were characterized by sharp zones for the investigated mixture and not sharp zone for the terminating electrolyte.

Time of analysis was changed depending on the number of compounds in the analyzed samples. The shortest optimum time of analysis (at about 130 sec)

TABLE 6 Characteristic of Used Analytical Method

Parameter	Unit	For Examined Ion
Precision[a]	%	2–4
Recovery[b]	%	91 ± 3
Linearity[c]	$mg \cdot mL^{-1}$	3–35
Limit of identification[d]	$mg \cdot mL^{-1}$	1

[a]$n = 5$, the samples were analyzed twice.

[b]The sample was enriched with 1.5 mL of a solution containing $1\ mg \cdot mL^{-1}$of examined ion, $n = 5$.

[c]Correlation coefficient above 0.98.

[d]Calculated from the limit of identification and coefficients of the calibration curve.

was obtained for one derivative (Table 3, Figs. 3 and 4). On the other hand, the optimum time of analysis was extended over two-fold (Table 5, Fig. 5).

Besides selection of a proper pH, it was necessary to choose adequate current intensity and time for individual steps of analysis. Finally, selection of these three parameters enabled optimum resolution of analyzed isomers (Fig. 5).

Investigations of isomer mixtures by the ITP technique were carried out using two dimensional analyses with switching of column. A; qualitative analysis was performed on the basis of heights of the zones. Obtained results were compared with isotachophoregrams of standard solutions.

The ITP method was characterized by high precision and accuracy of obtained results (Table 6). Linearity of the method was between 3 and $35\ mg\ L^{-1}$, detection limit was $1\ mg\ L^{-1}$.

To sum up, the aim of our investigation has been fully executed, i.e., optimum conditions of isotachophoretic separation and determination of chosen hydroxystilbazoles. Chromatographic investigation concerning this group of compounds and their derivatives involved numerous difficulties, independently on selection of analytical technique.[16–20] However, for reasons of biological activity of these compounds and wide possibilities of their applications, research in this area seems to be justified. These investigations are especially important because of antimicrobial and anticancer activity of hydroxystilbazoles.

CONCLUSIONS

Optimum conditions of isotachophoretic separation and determination of two isomers: (E)-N-(benzyl)-4′-hydroxystilbazole bromide and (E)-N-(benzyl)-2′-hydroxystilbazole bromide, have been elaborated. For the successful determination, we used a terminating electrolyte consisting of an aqueous solution of 4,4′-bis{1-(perhydroaminiomethyl)spirobi(1-sila-2,5-dioxacyclopentan-3-on)]at}. Elaboration of optimum conditions

confirms the opinion that hydroxystilbazoles can be successfully analyzed by the ITP technique.

ACKNOWLEDGMENTS

The authors are cordially indebted to Prof. N. Erchak for making available the use of 4,4′-bis{1-(perhydroaminiomethyl)spirobi(1-sila-2,5-dioxacyclopentan-3-on)]at}, which was the main component of the terminating electrolyte.

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